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# EFFECTS OF LONG-CHAIN FATTY ACIDS ON CULTURE DYNAMICS IN HYDROGEN FERMENTATION

Noori Saady  
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# **EFFECTS OF LONG-CHAIN FATTY ACIDS ON CULTURE DYNAMICS IN HYDROGEN FERMENTATION**

by

Noori M. Cata Saady

A Dissertation

Submitted to the Faculty of Graduate Studies through  
the Department of Civil & Environmental Engineering  
in Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy at the  
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2011

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CULTURE DYNAMICS IN HYDROGEN FERMENTATION**

by

Noori M. Cata Saady

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## **Declaration of Co-Authorship / Previous Publication**

**I. Co-Authorship Declaration:** I hereby declare that this thesis incorporates material that is the result of joint research:

1- It incorporates the outcome of laboratory work, which was performed by Dr. Subb Rao Chaganti under the supervision of Dr. Daniel Heath; the contribution by the co-authors is limited to the provision of microbiological results which are included in Chapters 5, 6, 7, and 8 of the dissertation.

2- Assistance with the physical work in the laboratory for the studies described in Chapters 7 and 8, except for the maintenance of the culture, preparation of the batch serum bottles, and the long-chain fatty acids extraction, was partially provided by Saravanan R. Shanmugam and Sathyanarayanan S. Veeravalli.

In all cases, the key ideas, primary contributions, experimental designs, engineering laboratory work, data analysis and interpretation, were performed by the author under the guidance of Dr. Jerald A. Lalman.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis.

I certify that, with the above qualifications, this thesis, and the research to which it refers, is the product of my own work.

**II. Previous Publication:** I hereby certify that no part of this thesis has been published or submitted for publication.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

## ABSTRACT

The effects of long chain fatty acids (LCFAs) on H<sub>2</sub>-producing mixed anaerobic cultures were evaluated using kinetic, biochemical, molecular biology and statistical methods. The objectives of this study were to assess changes in microbial diversity and metabolic pathways by exposing the cultures to LCFAs. Batch experiments were conducted at 37°C and initial pH 5.0 containing a mixed culture fed glucose and LCFAs (linoleic, palmitic, myristic, lauric, or a 50:50 mixture of myristic and palmitic acids). The H<sub>2</sub> yields for cultures from different sources were statistically the same when LA was added (3.3 mole H<sub>2</sub>·mole<sup>-1</sup> glucose; conversion efficiency = 82.5%). LA inhibited H<sub>2</sub>-consumption, propionate production, and directed the electron flux towards acetate:butyrate fermentation. *Clostridia*, *Bacteroides*, *Flavobacteria*, *Syntrophus*, *Geobacillus*, *Actinobacillus*, *Citrobacter*, *Cytophaga*, *Enterobacter*, *Erwinia*, *E. coli*, and *Klebsiella* dominated the LA-treated culture while *Bacteroides*, *Bacillus*, *Clostridia*, *Acinetobacter*, *Falvobacteria*, *Eubacteria*, and *Rubrobacter* dominated the control culture.

When LA was added at an initial pH of 5.0, the H<sub>2</sub> specific consumption rate decreased by approximately 99%; however, only a 24% reduction was detected in controls when the initial pH was set at pH 5.0. In the presence of LA, acetate, propionate, and butyrate degradation were inhibited.

LA and its degradation by-products (PA, MA, and LUA) sustained higher H<sub>2</sub> yields for 30 days in suspended cultures but not in granulated cultures. LA affected the diversity of the microbial communities differently in suspended and granular cultures from the same

origin. After LA-treatment, the suspended culture showed a greater diversity of acidogens than the granular culture.

Adding PA increased the H<sub>2</sub> yield; however, LCFAs shorter than 16 carbons did not show any H<sub>2</sub> production. *Thermoanaerovibrio*, *Geobacillus* and *Eubacteria* dominated the control cultures while *Clostridia* comprised less than 1% of the biomass. LCFAs caused an increased in the abundance of *Clostridia* and *Bacillus* to differing degrees (PA<MA<MA:PA<LUA). PA decreased the amount of *Thermoanaerovibrio*, while increasing levels of *Geobacter lovely* and *Bacteroides* were detected.

Long-term adaptation to glucose eliminated *Bacteroides*, *Parabacteroides*, *Lactobacillus*, *Fusobacterium* and *Syntrophobacter*, and reduced the abundance of *Deslfovibrio* and *Desulfobacter*. H<sub>2</sub>-consumption decreased and H<sub>2</sub> yield increased following LA-treatment and long-term adaptation to glucose.

## THESIS ORGANIZATION

Chapter 1 introduces the topic of hydrogen ( $H_2$ ) production using inhibited mixed anaerobic cultures. Culture dynamics are examined to determine the role of inhibitory agents such as long-chain fatty acids (LCFAs) in controlling the growth of  $H_2$ -consuming microorganisms. The research objectives pertaining to each phase of the experimental work are presented in Chapter 1. Chapter 2 reviews the important parameters, processes, and concepts relevant to the engineering, kinetic, and microbiological aspects of  $H_2$  production, and optimization using biochemical and statistical methods. Using published research findings and experimental data, this chapter provides details which are used to relate the microbiology of  $H_2$  production and  $H_2$  consumption in anaerobic communities. Information related to the inhibition and degradation of LCFAs is also provided in this chapter.

Chapter 3 describes in detail the materials, protocols, analytical methods, and experimental designs used in this study. The results of this research are reported in Chapters 4 to 8. The effects of linoleic acid (LA) on three cultures with distinct characteristics and from different sources are discussed in Chapter 4. Chapter 5 focuses on characterizing the different cultures using kinetic, microbiological, and statistical methods. It also investigates the effects of long-term adaptation of the cultures to glucose on the culture composition, activity and diversity. Chapter 6 describes a study designed to elucidate and evaluate the effects of linoleic acid and low initial pH on the population concentration and specific activity of various trophic groups comprising the active biomass, as well as the diversity of the mixed culture, with a focus on  $H_2$  consumption. A study to monitor and evaluate the effects of lauric, myristic, and palmitic acids on  $H_2$

production in mixed culture and to assess their effects on the diversity of the culture is presented in Chapter 7. The effects of varying linoleic acid incubation periods, linoleic acid degradation by-products, and the culture's physical structure on H<sub>2</sub> production in suspended and granular cultures are described in Chapter 8. Chapter 9 summarizes the research from studies presented in Chapters 4 to 8. Chapter 9 also integrates the work described in Chapters 4 to 8 to the broader field of H<sub>2</sub> dark fermentation and general conclusions are also presented. Chapter 10 discusses the engineering significance of this research to the field of study. Suggested potential applications for research findings, and directions for future research are also described in Chapter 10.

## **DEDICATION**

I dedicate this dissertation to my beloved and wonderful family:  
my parents, Zainab, Ahmad and Aya  
for all of their love and for always being supportive of my choices.

*Zainab*, you have been with me every step of the way, through good times and bad. Thank you for all of the support and for your decision to postpone your studies in order to look after the kids and me. Thank you very much.

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All good things that may come out of this dissertation I must share in one way or another with all of those I have indicated above; however, any errors in style or substance are my sole responsibility.

Noori Saady



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## NOMENCLATURE

$P_{H_2}$	Hydrogen partial pressure
16S rRNA	16S ribosomal RNA
acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AFBR	Anaerobic fluidized bed reactor
ASBR	Anaerobic sequencing batch reactor
ATP	Adenosine triphosphate
BESA	2-bromoethanesulfonic acid
C10	Capric acid
C12	Lauric acid
C14	Myristic acid
C16	Palmitic acid
C18:0	Steric acid
C18:1	Oleic acid
C18:2	Linoleic acid
C4	Butyric acid
C6	Caproic acid
C8	Caprylic acid
CH <sub>4</sub>	Methane
CIGSB	Carrier-induced granular sludge bed
COD	Chemical oxygen demand
CO <sub>2</sub>	Carbon dioxide
H <sub>2</sub>	Hydrogen
CSABR	Continuously stirred anaerobic bioreactor
CSTR	Completely stirred tank reactor
DGGE	Denaturing gradient gel electrophoresis
FBBAC	Fixed bed bioreactor with activated carbon
FBR	Fluidized bed reactor
Fd <sub>red</sub>	Reduced ferredoxin
Fe <sup>2+</sup>	Iron ion
FISH	Fluorescence in situ hybridization
H <sub>2</sub> /CO <sub>2</sub>	Hydrogen/carbon dioxide gas mixture
HCl	Hydrochloric acid
HRT	Hydraulic retention time
$k_a$	Maximum specific substrate utilization rate
K <sub>s</sub>	Half-saturation concentration
LA	Linoleic acid (C18:2)
LCFAs	Long chain fatty acids
LDH	Lactate dehydrogenase
LUA	Lauric acid (C12)
MA	Myristic acid (C14)
MBR	Membrane bioreactor
MCF	Mixed culture fermentation
FBA	Flux balance Analysis

Mg <sup>2+</sup>	Magnesium
MIC	Minimum inhibition concentration
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide Adenine Dinucleotide
NaOH	Sodium hydroxide
OA	Oleic acid (C18:1)
PA	Palmitic acid
pHi	The internal pH of the cell
pK <sub>a</sub>	Acid dissociation constant
PCA	principal components analysis
PLFAs	Phospholipid fatty acids
PMMA	Polymethymethacrylate immobilized cells,
PTA	Phosphotransacetylase
SRB	Sulfate reducing bacteria
T-RFLP	Terminal restriction fragment length polymorphism
TS	Total solids
UASB	Upflow anaerobic sludge blanket reactor
VFAs	Volatile fatty acids
V <sub>max</sub>	Maximum hydrogen uptake velocity
VS	Volatile solids
VSS	Volatile suspended solid
X <sub>a</sub>	Percentage of active trophic group
ΔG <sup>o</sup>	Standard free energy
ΔpH	pH difference between inside the cell and growth medium

# CHAPTER 1: INTRODUCTION

## 1.1 BACKGROUND

Energy supplies are an essential requirement for supporting life and economic development. Increasing global consumption, cost, and security of energy services are major global concerns and energy demand is expected to increase by more than 50% by 2030 (Ball and Wietschel, 2009). Increased levels of carbon dioxide (CO<sub>2</sub>), global warming, and associated climate change are directly related to the increasing energy demand and the continuing dependence on fossil fuels (85% of all energy sources) (Das and Veziroglu, 2001). Consequently, balancing energy usage, environmental impacts, and economic growth is a task in reducing the usage of fossil fuels. The effect on the environment is becoming a controlling factor that may limit fossil fuel-associated economic growth. Therefore, energy needs and environmental concerns are driving the search for new pollution-free alternatives for renewable and sustainable energy supplies.

Among the alternatives available, hydrogen (H<sub>2</sub>) is an optimal candidate for the next energy carrier. Hydrogen can be produced and consumed by pollution-free processes; it has a high calorific value (3042 cal m<sup>-3</sup>) that is 2.75 times greater than that of hydrocarbon fuels (Das and Veziroglu, 2001). Production of large quantities of H<sub>2</sub> faces challenges, however, in order to be cost-effective, renewable, and environmentally-friendly (Melis and Melnicki, 2006).

Biological methods of H<sub>2</sub> production are sustainable and have minimal energy requirements (Das and Veziroglu, 2001). One of these, the dark fermentation of carbohydrates to produce H<sub>2</sub> economically, has immediate potential and commercial feasibility because it combines waste reduction with high H<sub>2</sub> production and can use the same hardware as industrial CH<sub>4</sub> fermentation (Benemann, 1996; Wu *et al.*, 2008).

Large-scale sustainable H<sub>2</sub> production using dark fermentation requires renewable feedstock chemicals and inocula. Low-value feedstocks rich wastes from agriculture and the food industry are suitable substrates (Hawkes *et al.*, 2007) while mixed anaerobic cultures are cheap, easy to obtain and can maintain inocula that can utilize non-sterile feedstock (Hawkes *et al.*, 2007). H<sub>2</sub> production from mixed cultures is economical because contamination is not a problem. This is very important because heat treatment of the inoculum at an industrial scale is technically difficult and the cost effectiveness, of any of the complex technical solutions, has not been demonstrated to date (Hawkes *et al.*, 2007).

Microbes catalyze the production of H<sub>2</sub> from biomass; therefore, the microbial communities involved could impact the process performance (Ziganshin *et al.*, 2010). The yield of H<sub>2</sub> from dark fermentation depends on both the H<sub>2</sub>-producing and the H<sub>2</sub>-consuming microorganisms (Hawkes *et al.*, 2007). An inherent problem in H<sub>2</sub> production by mixed culture is the rapid consumption of H<sub>2</sub> by microorganisms such as hydrogenotrophic methanogens and homoacetogens. Hydrogenotrophic methanogens and homoacetogens produce methane (CH<sub>4</sub>) and acetate from H<sub>2</sub> and CO<sub>2</sub>, respectively. Although the maximum theoretical H<sub>2</sub> yield is 4 mole H<sub>2</sub>·mole<sup>-1</sup> hexose assuming acetate is the only reduced carbon by-product, reported yields vary from 0.57-2.2 mole H<sub>2</sub>·mole<sup>-1</sup> hexose (Hallenbeck and Ghosh, 2009; Logan *et al.*, 2008) because of the activity of H<sub>2</sub>-consuming microorganisms. Converting H<sub>2</sub> to CH<sub>4</sub> results in a net loss of energy because the energy content of CH<sub>4</sub> is only 42% of that of H<sub>2</sub> on the basis of mass. Only 77% of the energy in H<sub>2</sub> is recovered in CH<sub>4</sub> (Eq. 1.1), therefore, decreasing H<sub>2</sub> consumption is crucial to the development of practical H<sub>2</sub> production applications (Lay *et al.*, 1999; Hawkes *et al.*, 2007).



Many pretreatments and toxic chemical inhibitors have been investigated as potential methanogenic inhibitors and subsequently, increase the  $H_2$  yield in mixed cultures. However, none to date have been proven practical for large-scale implementation and cost effectiveness. Long chain fatty acids (LCFAs) can replace toxic chemical inhibitors of acetoclastic and hydrogenotrophic methanogenesis (Hanaki *et al.*, 1981; Koster and Cramer, 1987; Lalman and Bagley, 2000). LCFAs are biodegradable and environmentally friendly organics that can be obtained from natural sources such as the waste stream of some food-processing industries (Lalman and Bagley, 2002). Thus, they could be the most economical as well as effective inhibitors, and would support a greener process.

Mixed cultures are composed of a consortium of several distinct functional trophic groups of microorganisms, which together degrade the organic substrate to  $CH_4$  and  $CO_2$  through their metabolic activities. To date, no studies investigating the behavior of the various trophic groups in anaerobic mixed cultures and the relationship with  $H_2$  production have been published. Complete understanding of the microbial ecology of  $H_2$  production by dark fermentation is essential for effective and reliable control of the process.

Although  $H_2$  consumption was observed during previous  $H_2$  production studies insufficient attention has been given to the kinetic (activity) and microbiological aspects of the  $H_2$  consumers. Moreover, there is a lack of information on the kinetics of the various trophic groups under various pretreatment methods and inhibitors used in  $H_2$  production studies.

This research integrates engineering, kinetics, microbiology, biochemical modeling, and statistical methods to investigate the dynamics of mixed culture in  $H_2$  production by dark fermentation under the inhibition of LCFAs and low pH; specifically, the

effects of LCFAs on the various functional trophic groups of mixed culture are assessed.

The objective of this research is to assess mixed microbial communities and how they interact under different conditions by addressing four fundamental aspects of microbial communities: 1) function (reactions executed by community members); 2) phenotypic diversity; 3) structure (granular versus suspended/flocculent); and 4) inter-relationships among the community members (affecting production/consumption of H<sub>2</sub> and other metabolites). Consequently, kinetic, metabolic, molecular biology, and statistical methods were applied to monitor the microbial communities and to follow electron fluxes in these communities while under stress from exposure to LCFAs and low pH during dark fermentation H<sub>2</sub> production processes.

## **1.2 OBJECTIVES OF THE STUDY**

The objectives were to study the effects of LCFAs on H<sub>2</sub> production and the dynamics of mixed anaerobic cultures at 37 °C with an initial pH 5.0; in particular, to assess the impact of several LCFAs, low pH, and long-term adaptation to glucose on different types of cultures (acidogenic and methanogenic) from different sources (industrial and municipal). The work focused on assessing changes in the composition and diversity within the microbial community, population and activities of the various active trophic groups in the mixed anaerobic culture. To achieve these objectives, the research was divided into five phases:

### **Phase I: Effects of linoleic acid (LA, C18:2) on H<sub>2</sub> production in cultures from different sources**

LCFAs are inhibitory to anaerobic mixed cultures; however, it is still unclear if their effect on H<sub>2</sub> production varies from culture to culture. This phase assessed the effects of LA on H<sub>2</sub> production from glucose in three cultures (two methanogenic and

one acidogenic) from different sources (reactors treating wastewater from municipal, brewery, and ethanol manufacturing plants) in batch reactors studies at 37°C with an initial pH 5.0. The cultures' performance was compared using a multivariate statistical method and flux balance analysis (FBA).

### **Phase II: Effects of long-term adaptation to glucose on culture structure, composition, and activity**

This phase compared the three cultures examined in phase I using substrate kinetic and molecular biology terminal restriction fragment length polymorphism (T-RFLP) methods before and after twelve months of glucose adaptation to determine the effect of long-term adaptation on the size and activity of the various trophic groups in the microbial community. The stability of H<sub>2</sub> production and yield after long-term adaptation to glucose was tested. The kinetic protocol determined the concentration of active biomass and its metabolic activity (maximum specific substrate utilization rate) for several trophic groups (glucose, lactate, acetate, propionate, butyrate, and H<sub>2</sub> degrading microorganisms) in the mixed culture; hence, kinetic methods were used to determine the composition of the culture while molecular biology methods (T-RFLP) defined the diversity of the microorganisms present.

### **Phase III: Effects of linoleic acid and pH on the population and activity of the various trophic groups and H<sub>2</sub> yield in mixed culture.**

In Phase III, the effects of lowering the pH from 7.6 to 5.0 followed by the addition of 2,000 mg L<sup>-1</sup> LA on the concentration of active biomass and its maximum specific activity for various trophic groups (glucose, lactate, acetate, butyrate, propionate, and H<sub>2</sub> degrading microorganisms) were evaluated. The microbial diversity was analyzed using T-RFLP with a focus on H<sub>2</sub>-consuming bacteria.

#### **Phase IV: Effects of several LCFAs (shorter than C18) on H<sub>2</sub> production and the diversity of mixed anaerobic culture**

This phase evaluated and compared the influence of lauric (LUA, C12:0), myristic (MA, C14:0), palmitic (PA, C16:0), and a mixture of 50:50 myristic:palmitic (MA:PA) acids on H<sub>2</sub> production and the diversity of mixed anaerobic culture at 37°C with an initial pH 5.0 in batch reactors. The purpose of this phase was to find potential green methanogenic inhibitors and enhance the H<sub>2</sub> yield in dark fermentation. Such a finding could make the process more economical. Principal components analysis (PCA) and flux balance analysis (FBA) were used to compare the metabolic performance and diversity of the cultures treated with the different LCFAs.

#### **Phase V: Effects of incubation period, linoleic acid degradation by-products, and sludge physical structure on H<sub>2</sub> production from glucose.**

Linoleic acid is degraded by anaerobic culture to shorter LCFAs, acetate, and H<sub>2</sub>; the time frame for complete degradation is one order of magnitude greater than the time required to ferment glucose to H<sub>2</sub>. This phase examined and compared the effects of the incubation period (0 to 25 days) and LA degradation by-products on H<sub>2</sub> production and microbial diversity in suspended and granular cultures. PCA and MFA were used to compare the metabolic performance and diversity within the cultures.

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## CHAPTER 2: LITERATURE REVIEW

### 2.1 INTRODUCTION

Important parameters, processes, concepts, and published research findings related to hydrogen ( $H_2$ ) production using mixed culture dark fermentation are reviewed in this chapter. Engineering, kinetic, and microbiological aspects of  $H_2$  production are emphasized.

Hydrogen is produced during dark fermentation (anaerobic digestion) by acidogens and acetogens, and it is subsequently consumed by hydrogenotrophic methanogens, homoacetogens, and sulfate-reducing bacteria to maintain thermodynamic stable conditions. The process of  $H_2$  production by dark fermentation is described with a focus on the microbial aspects, metabolic pathways and biochemical reactions involved in each step; special attention is given to the role of homoacetogens. The microbiology of  $H_2$  -producing,  $H_2$  -consuming, and LCFA-oxidizing bacteria is then highlighted. The effects of important process and engineering parameters such as pH, temperature,  $H_2$  partial pressure ( $P_{H_2}$ ), volatile fatty acid by-products, hydraulic detention time (HRT), type of bioreactor, and pure and mixed substrates are explained.

Improving  $H_2$  yield during mixed culture dark fermentation is particularly important if the process is to become economically feasible. Techniques suggested to minimize  $H_2$  consumption and treatment methods used to inhibit methanogens and enrich  $H_2$  producers are reviewed. Long-chain fatty acids (LCFAs) are presented as alternative inhibitors that

could direct the electron flux towards  $H_2$  instead of methane ( $CH_4$ ); their uptake, toxicity, and degradation by mixed anaerobic cultures are presented.

The kinetics of the various trophic groups in mixed culture dark fermentation are described through their specific activities, proportions, substrate acclimation, and effects on the culture source. Terminal restriction fragment length polymorphism (T-RFLP) is the molecular biology technique used to identify the species of mixed cultures present under different conditions during  $H_2$  production. Principal component analysis (PCA), the multivariate statistical method used in the analysis of the engineering and microbiological results, is also described.

## **2.2 ANAEROBIC DIGESTION**

Anaerobic digestion is a well developed process for wastewater treatment and other biotechnology industrial applications. In this process, reduced organic materials are degraded by a series of biochemical reactions through the metabolic interactions of various anaerobic microorganisms (Figure 2.1). Four major groups of microorganisms work together to share the energy for growth and produce methane, carbon dioxide and water in four successive stages (Batstone *et al.*, 2002). The process depends on a balanced symbiotic relationship between metabolically distinct microbial groups (Plaza *et al.*, 1996). The main groups of microorganisms are (McInerney and Bryant, 1981b):

1. Hydrolytic and fermentative (acidogenic) bacteria
2. Hydrogen-producing acetogenic bacteria
3. Methanogenic bacteria (both acetoclastic and hydrogenotrophic)
4. Homoacetogenic bacteria

Hydrolysis, acidogenesis, acetogenesis, and methanogenesis are the successive stages of anaerobic fermentation (Figure 2.1) (Angenent *et al.*, 2004). Each stage has a

physiologically unique microbial population. The four stages occur synergistically in a successive fashion such that the product of one reaction becomes the substrate for the next reaction. The various trophic groups differ in terms of physiology, nutritional needs, growth kinetics, and tolerance to environmental conditions (Pohland and Ghosh, 1971). For example, the optimum growth pH is 5.2 to 5.9 for acidogens (McCarty and Mosey, 1991), 6.0 to 7.0 for acetogens, and 7.5 to 8.5 for methanogens (Solera *et al.*, 2002).

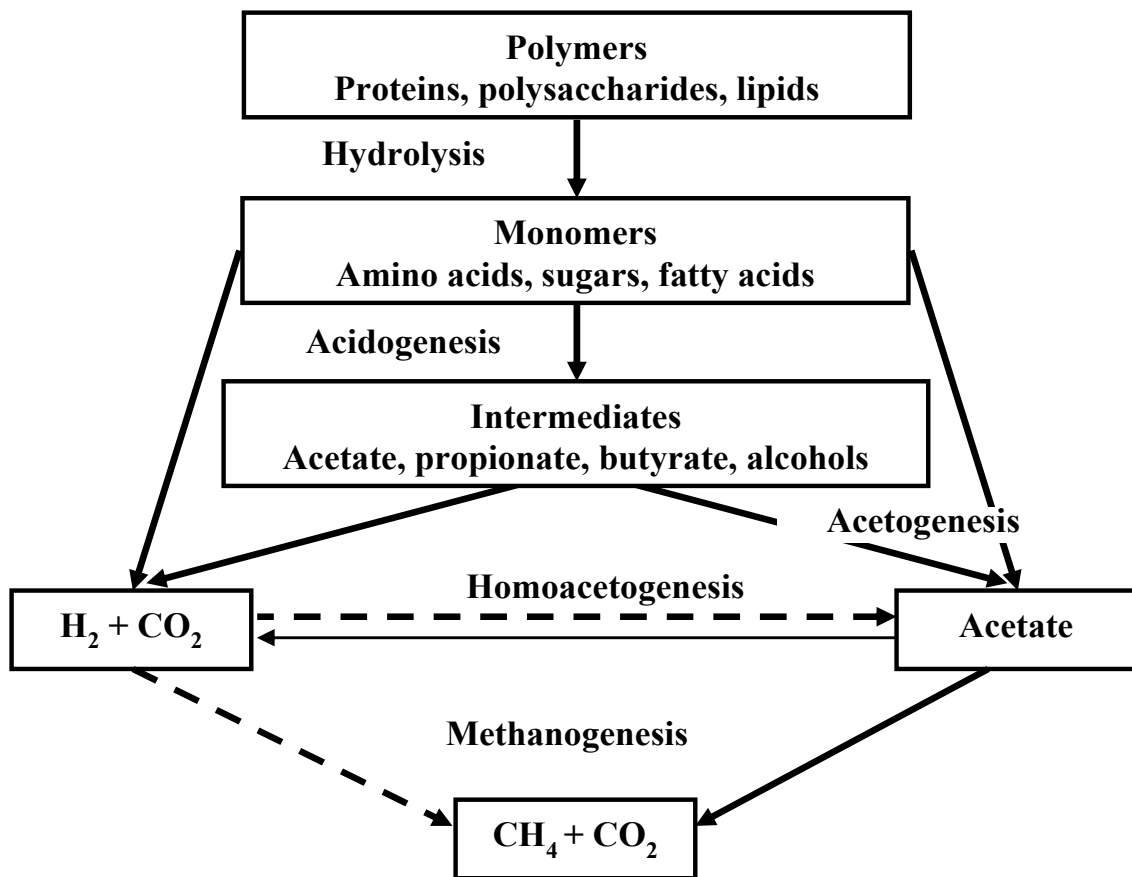


Figure 2.1 Anaerobic digestion process. (Adapted from Angenent *et al.*, 2004)

### 2.2.1 Hydrolysis or liquefaction

Hydrolysis is an extracellular process which converts large complex insoluble organic matter (polymers) into small simple soluble form (monomers) that can be transported into

the microbial cells and subsequently metabolized (Parkin and Owen, 1986). Extracellular enzymes (cellulase, cellobiase, xylanase, amylase, protease, and lipase) secreted by the microorganisms catalyze the hydrolysis of different polymers (Batstone, 2000).

The rate of hydrolysis depends on pH, temperature, composition and particle size of the substrate, concentration of the substrate, biomass concentration, enzyme production and adsorption, and the concentration of intermediate products (Batstone *et al.*, 2000). The hydrolysis produces dissolved products that are taken up and fermented by the fermentative/acidogenic bacteria. The hydrolysis of carbohydrates containing glucose, sucrose and starch is not rate limiting in anaerobic digestion (Zoetemeyer *et al.*, 1982).

### **2.2.2 Fermentation/acidogenesis**

Fermentation is a microbial process in which part of the organic molecule to be oxidized acts as a terminal electron acceptor in the absence of external electron acceptors such as nitrate or sulfate (Gujer and Zehnder, 1983). Carbohydrates, for example, are rapidly converted by hydrolysis to simple sugars, and subsequently fermented to volatile fatty acids (Mata-Alvarez, 2003).

Acidogens convert the simple monomers produced in hydrolysis into volatile fatty acids through fermentative reactions (Table 2.1: Eqs. 2.1 to 2.6). Acidogenesis is a complex process that produces volatile fatty acids (acetate, propionate, butyrate, lactate, caproate, valerate, heptate, etc.), hydrogen, alcohols, and carbon dioxide (Parawira, 2004). By-product formation and distribution in acidogenesis depend on the bacterial species as well as environmental conditions such as pH (Yu and Fang, 2003). Volatile fatty acids (VFAs) are the most common intermediates of fermentative bacteria in mixed culture anaerobic digestion (Pind *et al.*, 2003). VFAs produced in acidogenesis decrease

Table 2.1 Free energies of some anaerobic bioreactions.

Acidogenic reactions	$\Delta G^{\circ}$ (kJ)	Eq. No.
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	-206.0	2.1
$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	-254.0	2.2
$3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2CH_3COOH + 2CO_2 + 2H_2O$	-694.7	2.3
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	-279.4	2.4
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3COOH + 2H_2O$	-198.1	2.5
$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + H^+$	-225.4	2.6
$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	-164.8	2.7
$3CH_3CHOHCOOH \rightarrow 2CH_3CH_2COOH + CH_3COOH + HCO_3^- + H^+$	-165.0	2.8
$2CH_3CHOHCOOH + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2HCO_3^- + H^+ + 2H_2$	-56.3	2.9
<b>Acetogenic reactions</b>		
Propionate: $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$	+76.2	2.10
Butyrate: $CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	+48.4	2.11
Lactate: $CH_3CHOHCOOH + 2H_2O \rightarrow CH_3COOH + HCO_3^- + 2H_2$	-4.2	2.12
Ethanol: $CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	+9.6	2.13
Homoacetogenesis: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	-104.0	2.14
<b>Methanogenic reactions</b>		
Hydrogen: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135.0	2.15
Acetate: $CH_3COOH \rightarrow CH_4 + CO_2$	-31.0	2.16
Formate: $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-304.2	2.17
Methanol: $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-312.8	2.18
Ethanol: $2C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COOH$	-31.6	2.19
<b>Sulfidogenic reactions</b>		
$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$	-152.2	2.20

the pH to acidic levels (pH <6) (Lay *et al.*, 1998).

Important species of fermentative bacteria include strict and facultative anaerobes such as *Clostridia*, *Bacteroides*, *Enterobacte*, *Eschericia*, *Klebsiella*, *Erwinia*, *Salmonella*, *Serratia*, *Pseudomonas*, *Aeromonas*, *Shigella* (Madigan *et al.*, 2000), *Clostridia*, *Bifidobacteria*, *Lactobacillaceae*, *Streptococcaceae*, *Enterobacteriaceae* (Tanisho *et al.*, 1998), *Fusobacterium* (Kim and Gadd, 2008), *Peptococcus anerobus* (Verma, 2002), *Butyrivibrio* (Novaes, 1986), *Propionbacerium* (Madigan *et al.*, 2000; Verhulst *et al.*, 1985), *Citrobacter* (Oh *et al.*, 2003), *Bacillaceae* (Iyer *et al.*, 2004), *Eubacterium*,

*Butyribacterium*, *Methylophilum*, and *Staphylococcus* (Fang *et al.*, 2002a). For example, species of *Clostridia*, strict anaerobes, produce mixed acid upon fermenting carbohydrate substrate. Some sulfate-reducing bacteria (SRB) perform fermentative and acetogenic metabolism (Widdel and Hansen, 1992).

Acidogens have a higher growth rate than acetogens and methanogens (Liu and Gosh, 1997; Pavlostathis and Giraldo-Gomez, 1991). *Clostridia*, for example, grow five to ten times faster than methanogens (Van Ginkel *et al.*, 2001). Acidogens and acetogens are the hydrogen-producers while hydrogenotrophic methanogens are the main hydrogen-consumers in anaerobic digestion. Therefore, acidogens are very important to  $H_2$  fermentation.

### 2.2.3 Acetogenesis

Acetogenic bacteria degrade (oxidize) organic acids such as propionic, butyric, valeric acids and alcohols to acetate,  $H_2$ , and carbon dioxide ( $CO_2$ ); hence they produce the substrates (acetate and hydrogen) utilized by methanogens (Parawira, 2004). Acetogens are obligate proton reducing bacteria capable of releasing electrons in the form of both  $H_2$  and formate (Drake, 1994). According to several reports, these organisms can tolerate a wide range of environmental conditions (Novaes 1986; Parkin and Owen, 1986). Acetogenic reactions (Table 2.1: Eq. 2.10 to 2.14) decrease the pH to more acidic levels ( $\sim$ pH 5) (Lay *et al.*, 1998) and produce  $H_2$ . However, high  $H_2$  partial pressure ( $P_{H_2}$ ) is known to inhibit acetogenic bacteria (Kaspar and Wuhrmann, 1978).

Acetogens grow very slowly and depend on the activity of hydrogenotrophic and acetoclastic methanogens (Voolapalli and Stucky, 1999). Therefore, they survive in a syntrophic relationship with methanogens to maintain a low  $P_{H_2}$  ( $<10$  Pa) (Table 2.1: Eq.

2.10 to 2.14). This relationship establishes favorable thermodynamic ( $\Delta G < 0$ ) conditions (Schink, 1997; Stams, 1994) and according to Boone *et al.*, (1993) acetogens undergo inhibition under elevated  $H_2$  levels.

Many acetogens can reduce carbon dioxide and the one-carbon compounds to acetate (Ljungdahl, 1983). Most thermophilic acetogens such as *Acetoneema longum* are able to degrade glucose into acetate with a product yield as high as 90% (Wiegel, 1994). According to Kane and Breznak (1991) *Acetoneema longum* degrades glucose stoichiometrically into acetate via the acetyl-coenzyme. Some important acetogenic bacteria in anaerobic communities includes *Acetobacter*, *Syntrophobacter*, *Syntrophomonas*, *Clostridium mayombeii*, *Clostridium pfennigii*, *Acetoneema longum*, *Eubacterium limosum*, and *Butyribacterium rettgeri*.

#### **2.2.4 Homoacetogens**

Homoacetogens are strict anaerobes, fast growing and some are spore-forming organisms (Table 2.2) according to Schink (1994). The organisms grow autotrophically (on  $H_2$  and  $CO_2$ ) and/or heterotrophically (fermentation of organic compounds) to produce acetate (Dolfing, 1988; Drake, 1994). In addition, autotrophic homoacetogens produce significant amounts of ethanol (Tanner *et al.*, 1993). Homoacetogens can either oxidize or synthesize acetate depending on the external  $H_2$  concentration (Schink, 2002). The two-way inter-conversion between  $H_2$  and acetate catalyzed by homoacetogenic bacteria plays an important role in the methane formation pathway. Although the role of



Table 2.2 Examples of acetogenic and homoacetogenic bacteria.

Species	Temp.	pH	Gram type	Sopre-forming ability	Homo-acetogenesis	Reference
<i>C. formicoaceticum</i>	NR*	NR	+	+	+	Andreesen <i>et al.</i> , 1970
<i>Acetobacterium Woodii</i>	mesophilic	NR	NR	NR	+	Novaes, 1986
<i>C. thermoaceticum</i>	NR	NR	+	+	+	Drake, 1994
<i>Acetoanaerobium</i>	mesophilic	NR	+/-	NR	+	
<i>Acetobacterium</i>	27-30	NR	+	-	+	
<i>Acetitomaculum</i>	38	NR	+	-	+	
<i>Acetogenium</i>	66	NR	-/+	-	+	
<i>Acetohalobium</i>	38-40	NR	-	-	+	
<i>Acetonema</i>	30	NR	-	+	+	
<i>C. Morrela</i>	mesophilic	NR	+/-	+	+	
<i>Eubacterium</i>	mesophilic	NR	+	NR	+	
<i>Peptostreptococcus</i>	35-42	NR	+	-	+	
<i>Sporomusa</i>	30	NR	+ /-	+	+	
<i>Acetoanaerobium</i>	37	NR	+	-	+	Schink, 1994
<i>Eubacterium (Butyribacterium)</i>	30-37	NR	+	-	+	
<i>C. ljungdahlei</i>	NR	NR	+	+	+	
<i>C. thermoautotrophicum</i>	55-60	NR		+	+	
<i>Syntrophococcus</i>	mesophilic	NR	-/+	-	+	
<i>Clostridium drakei</i>	5-40	3.6-7.4	NR	NR	+	Gößner <i>et al.</i> , 2008
<i>C. aceticum</i>	mesophilic	NR	NR	+	+	Novaes, 1986
<i>C. autotrophicum</i>	NR	NR	NR	+	+	Cheong and Hansen, 2006
<i>Moorella thermoacetica</i>	NR	5.7-7.7	NR	NR	-	Drake and Daniel, 2004
<i>E. limosum</i>	NR	NR	NR	NR	+	Bloas <i>et al.</i> , 1993
<i>Acetogenium kivui</i>	NR	6.0-6.2	NR	NR	+	Ruyet <i>et al.</i> , 1984
<i>Acetonema longum</i>	NR	NR	NR	NR	NR	Kane and Breznak, 1991

\* NR = not reported

homoacetogens in dark  $H_2$  fermentation is important, their role is still unclear (Ryan *et al.*, 2008).

Homoacetogens participate and compete in any anaerobic process in any environment because they are physiologically very versatile (Schnik, 1997). They compete with hydrogeotrophic methanogens at low pH or low temperature (Schink, 1997; Weijma *et al.*, 2002). At high  $P_{H_2}$  (>500 Pa), acetogenesis is favored (from  $H_2$  and  $CO_2$ ) and at low  $P_{H_2}$  (<40 Pa), acetate oxidation occurs (Demirl and Scherer, 2008).

*Clostridium* and *Acetobacterium sp.* are the most common autotrophic and heterotrophic homoacetogenic genera (Ryan *et al.*, 2008). For example, *C. magnum*, *C. thermoaceticum*, and *C. thermoautotrophicum* convert glucose or sucrose to acetate and can also grow autotrophically on  $H_2/CO_2$  to produce acetate (Minton *et al.*, 1989).

### 2.2.5 Methanogenesis

Methanogenesis is the final stage of anaerobic digestion. Methanogens are fastidious, slow-growing, strict anaerobes. They are sensitive to oxygen concentration (Zeikus, 1980) and require vitamins, unusual trace minerals (Co, Ni), fatty acids (acetate) or specific cofactors (coenzyme M) (Solera *et al.*, 2002). Methanogens are also sensitive to changes in operational parameters and environmental conditions such as pH, temperature, hydraulic loading rate, organic loading rate, and feed composition (Fey and Conrad, 2000; McHugh *et al.*, 2003; Rozzi *et al.*, 1994).

Methanogens produce methane either by cleavage of acetic acid molecules to  $CO_2$  and methane, or by reduction of  $CO_2$  with  $H_2$  (Table 2.1). They grow directly on acetate,  $H_2/CO_2$ , and other one-carbon compounds such as formate, methanol and methylamine (Schink, 1997; Stams, 1994). Acetoclastic methanogens can utilize only

acetate, while hydrogenotrophic methanogens use  $H_2/CO_2$ . Therefore, they live in syntrophic association with acetogens. Some methanogens grow on both  $H_2/CO_2$  and formate and some species can utilize  $H_2/CO_2$ , acetate, methanol and a few other one-carbon compounds (Stams *et al.*, 2005). In anaerobic digestion, up to 70% of methane is produced from acetate while 30% is derived from  $H_2/CO_2$  (Jeris and McCarty, 1965).

Methanogens affect both acetogens and acidogens by utilizing  $H_2$  and acetate (Stams and Zehnder; 1990). Hydrogenotrophic methanogenesis occurs simultaneously and syntrophically with acetogenesis. Hydrogeotrophic methanogens regulate the anaerobic process by maintaining low  $P_{H_2}$ , affect the syntrophic acetogens, and influence the whole fermentation process (Schink, 1997).

Hydrogenotrophic methanogens are more resistant to environmental changes and grow faster than acetoclastic methanogens (Parawira, 2004). The minimum doubling time of hydrogenotrophic and acetoclastic methanogens are 6 h and 1.5 to 2.6 days, respectively (Mosey and Fernandes, 1989). Acetoclastic methanogens have a slower growth rate because they produce lower free energy levels (Cummings and Stewart, 1995). The metabolic rates of acetoclastic methanogens are lower than those of acid-forming bacteria (Mosey and Fernandes, 1989). Therefore, methane production is generally the rate-limiting step in anaerobic digestion (Speece 1996). Inhibition of methanogens by low pH or toxic chemicals results in the accumulation of  $H_2$  and VFAs; such conditions increase concentrations of reduced products (Schink, 2002).

## **2.3 MICROBIOLOGY OF HYDROGEN PRODUCTION**

### **2.3.1 Hydrogen-producing bacteria**

In dark fermentation, different species of strict and facultative fermentative anaerobic bacteria (some have been reviewed in section 2.2.2 and Table 2.3) produce

Table 2.3 H<sub>2</sub> production in pure culture studies.

Microorganism	Substrate	pH	Temp (°C)	H <sub>2</sub> yield (mol·mol <sup>-1</sup> Substrate)	Reference
<i>C. butyricum</i>	Glucose continuous	6.7	30	1.3-2.0 1.4-2.0	Suzuki <i>et al.</i> , 1980 Karube <i>et al.</i> , 1982
<i>Enterobacter cloacae</i> DM11	Glucose continuous	5.5	36	3.8	Kumar and Das, 2001
<i>Enterobacter aerogenes</i> E82005	Molasses	6.0	38	1.58	Tanisho <i>et al.</i> , 1998
<i>Aciduric Enterobacter aerogenes</i>	Glucose	6.5	38	1.0	Yokoi <i>et al.</i> , 1995
<i>Enterobacter aerogenes</i>	Glucose	7	37	1.1	Rachman <i>et al.</i> , 1998
<i>Rhodopseudomonas palustris</i> P4	Glucose	5-9	25-40	2.76	Oh <i>et al.</i> , 2004
<i>Citrobacter</i> sp. Y19	Glucose Batch	7.0	36	2.49	Oh <i>et al.</i> , 2003
<i>C. tyrobutyricum</i>	Xylose	6.3	37	0.77	Zhu and Yung, 2004
<i>Escherichia coli</i>	Glucose Batch	6	37	3.12	Chittibabu <i>et al.</i> , 2006
<i>Caldicellulosiruptor saccharolyticus</i>	Xylose	NR	70	2.24	Kadar <i>et al.</i> , 2004
<i>Bacillus coagulans</i> IIT-BT S1	Glucose Batch	6.5	37	2.28	Kotay and Das, 2007
<i>Clostridium bifermentans</i>	Wastewater sludge	NR	33	0.25-0.36	Wang <i>et al.</i> , 2003a
<i>Citrobacter intermedius</i>	Glucose Batch	NR	34	1.0	Brosseau and Zajic, 1982
<i>C. pasteurianum</i>	Glucose Batch	NR	34	1.5	Brosseau and Zajic, 1982
<i>Bacillus licheniformis</i>	Glucose continuous	NR	NR	1.5	Kalia <i>et al.</i> , 1994
<i>C. acetobutylicum</i>	Glucose	6.0	37	2.0	Chin <i>et al.</i> , 2003
<i>C. butyricum</i> sp. strain no. 2	Xylose, batch	NR	31 36 41	2 2.3 2.4	Taguchi <i>et al.</i> , 1994
	glucose, batch	NR	31 36 41	2.5 2.8 2.8	Taguchi <i>et al.</i> , 1994
	Arabinose, batch	NR	31 36 41	2.2 2.3 2.4	Taguchi <i>et al.</i> , 1994

NR = not reported

H<sub>2</sub> during their metabolism. They are able to dispose excess electrons in the form of molecular H<sub>2</sub> through the activity of hydrogenase (Das and Veziroglu, 2001). The maximum yield of H<sub>2</sub> reported for pure culture of *Enterobacter cloacae* DM11 is 3.8 mol H<sub>2</sub>·mol<sup>-1</sup> glucose (Kumar and Das, 2001).

Strict anaerobes H<sub>2</sub> producers include *Clostridia*, methylophs, rumen bacteria, methanogenic bacteria, and archaea (Nandi and Sengupta 1998). Species of *Clostridia* are the most effective H<sub>2</sub> producers in dark fermentation (Levin, 2004; Das and Veziroglu, 2001); for example, *Clostridium butyricum* species can produce 2.4 mol H<sub>2</sub>·mol<sup>-1</sup> hexose (Taguchi, 1995). Other *Clostridium* species include *C. pasteurianum*, *C. butyricum*, and *C. beijerinckii* (Brosseau and Zajic, 1982; Lay *et al.*, 1999; Ueno *et al.*, 1995). *Clostridium* species (*Closteridiaceae*) constituted 64.6% of the clones detected in H<sub>2</sub> producing mesophilic mixed cultures whereas *Enterobacteriaceae* and *Streptococcaceae* constituted 18.8% and 3.1%, respectively (Fang *et al.*, 2002b). Heat treatment can be used to enrich mixed cultures with *Clostridia* because they are spore formers. Anaerobes such as *Actinomyces* sp. and *porphyromonos* sp. have been detected in addition to *Clostridia* in H<sub>2</sub> producing cultures (Kapdan and Kargi, 2006).

Facultative anaerobes include species such as *Escherichia coli* (Blackwood *et al.*, 1956), *Enterobacteriaceae* (Kumar and Das, 2001), *Citrobacter* (Oh *et al.*, 2003), and *Rhodopseudomonas* (Oh *et al.*, 2004), *Lactobacillaceae*, and *Bacillaceae* (Holt *et al.*, 1994; Nandi and Sengupta, 1998; Yu and Fang, 2001). According to Blackwood *et al.* (1956), engineered *E. coli* is able to produce 0.72 to 0.91 mol H<sub>2</sub>·mol<sup>-1</sup> glucose. Fang *et al.* (2002b) detected *Klebsiella* which is another H<sub>2</sub> producing microorganism found in mesophilic anaerobic communities. Work by Minnan *et al.* (2005) has shown

pure cultures of *Klebsiella oxytoca* HP1 can produce high levels of H<sub>2</sub> at 35°C. Generally, facultative bacteria produce less quantities of H<sub>2</sub> than strict anaerobes.

Spore forming bacteria constitute important H<sub>2</sub> producers. *Clostridia* and *Bacillus* both are spore formers and heat treatment results in their selection. Reports by Kalia *et al.* (1994) have shown *Bacillus licheniform* can produce yields up to 1.5 mol H<sub>2</sub>·mol<sup>-1</sup> glucose.

### 2.3.2 Hydrogen-consuming microorganisms

Hydrogen consumption has been reported in many studies; however, it has not been well quantified, or related to the activities of homoacetogenic, hydrogenotrophic methanogens, and sulfate reducers. Hydrogen consumption via acetogenesis during H<sub>2</sub> production from glucose has been observed in heat-treated culture (Sang *et al.*, 2003; Oh *et al.*, 2003) and in non-treated culture (Rossetti *et al.*, 2003). Kraemer and Bagley (2007) estimated H<sub>2</sub> consumption could account for 2-11% of the yield based on a measured H<sub>2</sub> consumption rate (0.34 mM/h) in batch reactors.

Many bacterial species are H<sub>2</sub>-consumers. *Lactobacillus* species are very common in H<sub>2</sub> fermentation and can affect the yield adversely. *Lactobacillus fermentoshensis* and *Lactobacillus paracasei* have been detected in H<sub>2</sub>-producing communities (Kawagoshi *et al.*, 2005). Lactic acid producers reported by Iyer *et al.* (2004) include *Bacillus racemilacticus* and *Bacillus myxolacticus* in heat-treated culture.

Some sulfate-reducing bacteria (SRB), such as *Desulfomicrobium*, *Desulfobulbus* and *Desulfobotulus* perform fermentative and proton-reducing acetogenic metabolism in addition to sulfate reduction (Widdel and Hansen, 1992; Raskin *et al.*, 1996). Moreover, *Desulfococcus*, *Desulfosarcina*, and *Desulfobotulus* groups can be very competitive for H<sub>2</sub> in the absence of sulfate (Raskin *et al.*, 1996).

*Desulfovibrio*-like bacterium predominates even in the absence of sulfate from the growth medium (Sousa *et al.*, 2007). *Desulfotomaculum geothermicum*, a spore forming H<sub>2</sub> consuming SRB, was detected in a thermophilic acidogenic culture, whereas *Thermotogales* strain was detected in a mesophilic acidogenic culture (Shin *et al.*, 2004). Gram-negative SRB such as *Desulfobacter*, *Desulfobacterium*, *Desulfonema*, *Desulfosarcina*, *Archaeoglobus* and *Desulforhabdus* consume H<sub>2</sub>/CO<sub>2</sub> to reduce sulphate autotrophically (Widdel and Hansen, 1992).

### **2.3.3 Pure versus mixed culture for H<sub>2</sub> production**

Economical H<sub>2</sub> production requires a cheap and sustainable process. Pure cultures are expensive and require sterilized substrate, while mixed cultures can use non-sterile substrates including those present in waste streams. Mixed cultures are available from wastewater treatment facilities, animal solid waste, compost, and other sources. The H<sub>2</sub> yields reported for mixed cultures (Table 2.4) do not differ substantially from that of pure cultures and, in some cases, are greater. Yields from glucose degradation by mixed cultures can range between 1.0 and 2.5 mol H<sub>2</sub>·mol<sup>-1</sup> glucose (Table 2.4). Hydrogen yields from mixed cultures utilizing carbohydrate, palm oil, and rice production facilities have been shown to reach maximum values from 1.95 to 2.52 mol H<sub>2</sub>·mol<sup>-1</sup> glucose. The major problem associated with mixed cultures is that they contain various H<sub>2</sub>-consuming microorganisms. Further research is required to reduce or eliminate H<sub>2</sub> consumption by methanogens and other microorganisms in mixed anaerobic cultures during H<sub>2</sub> fermentation.

Table 2.4 Hydrogen production in mixed culture studies.

Culture type	Substrate	pH	Temp (°C)	H <sub>2</sub> yield (mol·mol <sup>-1</sup> hexose )	Reference
Sludge compost	Cellulose	NR	60	2.4	Ueno <i>et al.</i> , 1995
Sludge compost	Sugary wastewater	6.8	60	2.52	Ueno <i>et al.</i> , 1996
Heat-treated anaerobic sludge	Glucose	5.5	33 35 37 39 41	0.97 1.0 1.22 1.57 1.67	Mu <i>et al.</i> , 2006
Mesophilic and thermophilic acidogenic culture	food processing wastewater	4.5 5.5 6.5 4.5 5.5 6.5	35    55	0.1 0.05 0.03 0.9 0.8 0.6	Shin <i>et al.</i> , 2004
Heat-treated soil	Glucose	5.5	30	2.5	Van Ginkel <i>et al.</i> , 2005a
Mixed bacterial flora	Rice winery wastewater	5	55	1.95	Yu <i>et al.</i> , 2002a
UASB	Chemical wastewater	6	30	0.21	Mohan <i>et al.</i> , 2007b
Cow dung	palm oil mill effluent	5	NR	2.03	Vijayaraghavan and Ahmad, 2006
Mesophilic sewage digested sludge	Glucose	NC	25	1.4	Lin and Chang., 2004
Sewage sludge	Xylose, Chemostat	7.1	30-55	0.4-1.4	Lin <i>et al.</i> , 2008
H <sub>2</sub> -producing sludge	Xylose, CSTR	NR	30-55	1.3	Lin <i>et al.</i> , 2008
H <sub>2</sub> -producing sludge	Glucose, Batch	NR	33-41	1.67	Chen <i>et al.</i> , 2006
H <sub>2</sub> producing sludge	Sucrose	NR	30-45	1.94	Lee <i>et al.</i> , 2006b
Compost	Xylose SBR	5	55	1.7	Calli <i>et al.</i> , 2008
Thermally-treated municipal sludge from final sedimentation tank	Starch	5-5.5	35	0.97-1.43	Wang and Chang, 2008
Compost	Lactose SBR	5 5.3	55	3.7 3.2	Calli <i>et al.</i> , 2008

NC = not controlled



### 2.3.4 LCFA-degrading microorganisms

Long chain fatty acids (LCFAs) are degraded to acetate and  $H_2$  via a  $\beta$ -oxidation mechanism by  $H_2$ -producing acetogens (Jeris and McCarty, 1965). Hydrogenation or saturation of unsaturated LCFAs can occur prior to  $\beta$ -oxidation (Figure 2.2) (Kuang, 2002). In some cases,  $\beta$ -oxidation of linoleic acid (LA) can proceed without complete saturation (Lalman and Bagley, 2000).

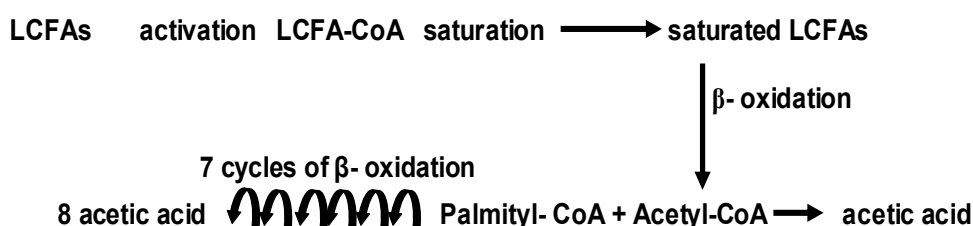
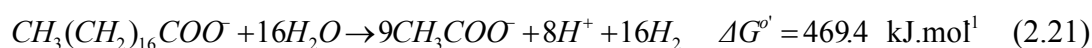


Figure 2.2 Degradation of LCFAs by  $\beta$ -oxidation reaction (Adapted from Kuang, 2002).

Under standard conditions, LCFA-oxidation is thermodynamically unfavorable. For example, the free energy of the complete degradation of linoleic acid to acetate and  $H_2$  is positive (Eq. 2.21); in order to be thermodynamically favorable, the  $P_{H_2}$  has to be maintained to very low levels. This condition prevails only during the syntrophic association of LCFA-oxidizing with hydrogen-consuming microorganisms such as hydrogenotrophic methanogens (Schink, 1997).



Novel acetogenic LCFA- (C4-C18:2) degrading bacteria have been identified during the last four decades; however, the total number is relatively small. Most of the identified microorganisms belong to *Syntrophomonadaceae* and *Syntrophaceae* and function in syntrophy with methanogens (Sousa *et al.*, 2007). McInerney *et al.* (2008) reviewed characteristics, growth conditions (pH, temperature), substrates used, and

isolation conditions of identified microorganisms. *Syntrophomonas wolfei* was the first characterized syntrophic butyrate-oxidizing bacterium (McInerney *et al.*, 1981). Hatamoto *et al.* (2007) detected *Deltaproteobacteria*, *Clostridia*, and *Bacteroides* species in fatty acid-degrading consortia under methanogenic conditions. The findings of these researchers suggest that diverse bacterial groups are active in the degradation of LCFAs. The microorganisms identified so far include among others: *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium* (Fujimoto *et al.*, 1993), *Ruminococcus sp.*, *Eubacterium sp.* (Kemp *et al.*, 1975), *Clostridium sp.*, *Propionibacterium sp.* (Verhulst *et al.*, 1985), *Nocardia cholesterolicum* (Koritala and Bagby, 1992), and *Syntrophus aciditrophicus* (Jackson *et al.*, 1999).

### **2.3.5 Uptake and transport of LCFAs into microbial cells**

The uptake of biomaterial by a bacterial cell is a two-phase process: an initial physicochemical interaction phase (adsorption) and a late molecular and cellular phase (An and Friedman, 1998). According to the model proposed by Nunn, (1986), LCFAs are transported across cell membranes, and are activated by acyl-CoA synthetase before entering the  $\beta$ -oxidation cycle. The process proceeds via four steps (Figure 2.3) (Mangroo *et al.*, 1995). The transport of LCFAs through the outer membrane, peptidoglycan layer, periplasmic space, and inner membrane are all mediated by transport proteins; FadL and Tsp mediate transport through the outer membrane and peptidoglycan layer, respectively (Azizan and Black, 1994). The uptake and transport of LCFAs into the cell requires an energy investment. Acyl-CoA synthetase activates free LCFAs into long chain acyl-CoA complexes using adenosine triphosphate (ATP).

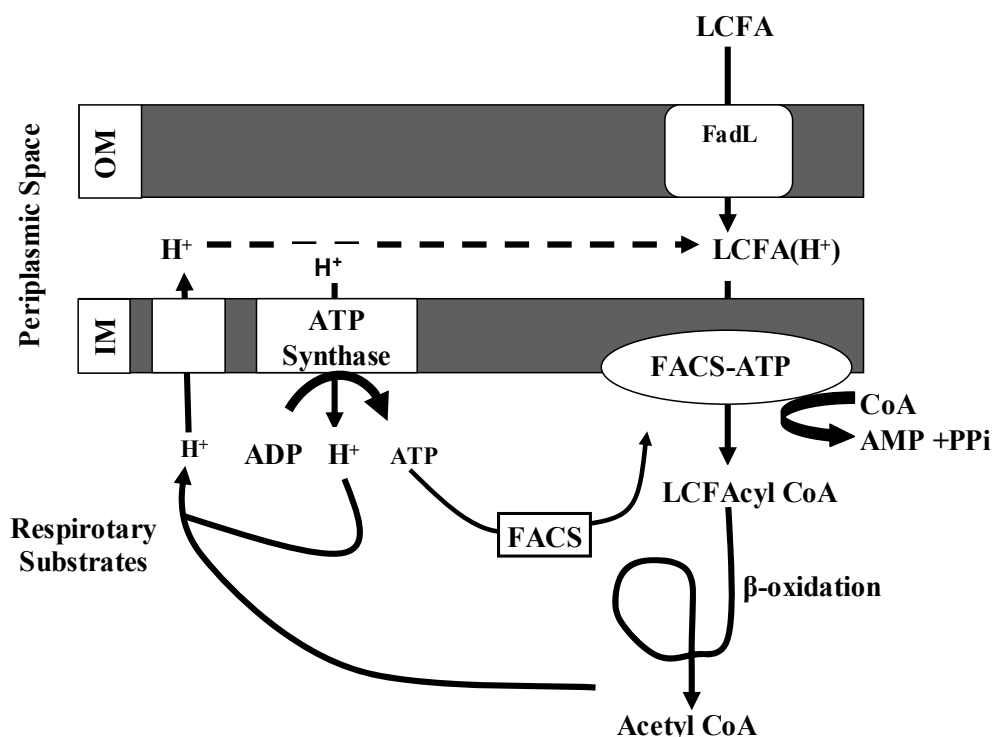


Figure 2.3 Proposed model for the transport of LCFAs (C12-C18) in *E. Coli*. (adapted from Azizan *et al.*, 1999). (OM and IM are outer and inner membranes of the cell, respectively).

### 2.3.6 LCFA toxicity and mode of action

LCFAs exert inhibitory effects even before they enter into a microbial cell. LCFAs disrupt membrane components and inactivate many reactions. Unsaturated LCFAs adsorb on the cell wall, alter its permeability, and limit or hinder the transport of substrates and nutrients (Demeyer and Henericks, 1967). They can also cause protein and ion leakage in Gram-positive bacteria (Galbraith and Miller, 1973).

Inside the cell, carboxylic acids dissociate, acidify the cytoplasm, and reduce the neutral internal pH of the cell. Consequently, they reduce the electro-motive potential and inactivate most enzymes (Madigan *et al.*, 2000), thus, affecting the synthesis of macromolecules such as DNA, RNA, proteins, and lipids (Cherrington *et al.*, 1991).

LCFAs inhibit the activity of various microorganisms of the anaerobic consortia (Hanaki *et al.*, 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1992;

Rinzema *et al.*, 1994; Lalman and Bagley, 2002) and decrease the availability of ATP (Hanaki *et al.*, 1981). Low concentrations of LCFAs inhibit Gram-positive but not gram-negative microorganisms (Nieman, 1954). Hydrolytic bacteria, acidogens, acetogens, and methanogens are affected to various degrees (Lalman and Bagley, 2002; Mykhaylov *et al.*, 2005). LCFAs influence hydrolytic and acidogenic bacteria by interfering with cellular functions responsible for glucose degradation (Lewis, 1992). LCFAs can inhibit acetogenic bacteria responsible for  $\beta$ -oxidation reactions that degrade the LCFAs themselves (Hanaki *et al.*, 1981). Methanogens are inhibited because their cell wall has a similar structure to Gram-positive bacteria (Ziekus, 1980, Madigan *et al.*, 2000).

Table 2.5 shows the reported minimum inhibitory concentrations of C8, C10, C12, C14, and C18:1 for several microorganisms; the susceptibility of the various

Table 2.5 LCFAs minimum inhibitory concentrations (MIC) for various bacteria.

	MIC (mM)					
	<i>Methanobrevibacter</i> sp.	<i>Bacillus megaterium</i>	<i>Pneumococcus</i> sp.	<i>Streptococcus</i> group A	<i>Streptococcus</i> beta-hemolytic. Non-group A	<i>Staphylococcus aureus</i>
<b>Fatty acid</b>						
C8:0	6.75	2.0	>6.9	>6.9	>6.9	>6.9
C10:0	2.6	1.0	1.45	1.45	2.9	2.9
C12:0	1.6	0.15	0.062	0.124	0.249	2.49
C14:0	2.6	0.15	0.218	0.547	2.18	4.37
C18:1	2.4	0.05	>3.5	1.77	>3.5	>3.5

(Adapted from Koster and Cramer, 1987).

microorganisms varied with the particular LCFA applied. Some studies suggest that a concentration of 1.0 g L<sup>-1</sup> LCFA can significantly inhibit anaerobic microorganisms (Hanaki *et al.*, 1981, Angelidaki *et al.*, 2002) but, other studies indicate that an

adaptation period could allow high LCFA concentrations (Cavaleiro *et al.*, 2001). LCFAs showed synergistic toxicity; for example, lauric acid can enhance the toxicity of capric and myristic acids (Koster and Cramer, 1987; Soliva *et al.*, 2004).

Adsorption, precipitation, and entrapment are the mechanisms by which LCFAs attach to the cell wall (Pereira *et al.*, 2004). Adsorption depends on the properties of the bacteria, biomaterial surface characteristics, and environmental conditions (Katsikogianni and Missirlis, 2004). The inhibitory effects of LCFAs are attributed to physical interactions between the acids (biomaterial) and the cell wall (Kodicek and Worden, 1945; Galbraith *et al.*, 1971; Demeyer and Henderickx, 1967). This has formed the basis by Rinzema *et al.* (1994). to consider the inhibition as a function LCFAs:biomass ratio Uncoupling agents interfere with protective functions and transport systems due to the adsorption of LCFAs (Galbraith *et al.*, 1973; Rinzema *et al.*, 1994). LCFAs alter the permeability of the LCFA-covered spots on the cell membrane and result in cell lysis (Galbraith *et al.*, 1971).

The layer of biomass-associated LCFAs imposes transport (diffusion) limitations which impedes access to the substrates as well as subsequent gas release (Pereira *et al.*, 2005). However, H<sub>2</sub> is transported fast and mineralized easily to methane under the inhibition of LCFAs because it is a small molecule (Pereira *et al.*, 2003).

LCFA toxicity is correlated with LCFA concentration (Koster and Cramer, 1978; Angelidaki and Ahring, 1995; Lalman and Bagley, 2001) and other characteristics e.g. number of carbon atoms and double bonds in the LCFA chain (Rinzema, 1988), and culture physical structure (surface area) (Hwu *et al.*, 1996). The molecular shape of unsaturated LCFAs is non-linear because of the C=C bonds. The degree of unsaturation causes more inhibition than saturated LCFAs (Kodicek and Worden, 1945).

The physical characteristics of the culture (specific surface area and size distribution) determine its susceptibility to inhibition by LCFAs. Granular sludge is more resistant to LCFAs than suspended and flocculent sludges which have a higher specific surface area (Hwu *et al.*, 1996). In addition, LCFAs cause physical instability to the biomass in studies conducted with continuous flow reactors (Rinzema *et al.*, 1994). According to work by Hwu *et al.*, (1998b) and Pereira *et al.* (2002) granular sludge flotation was observed at LCFA concentrations below the toxicity limit.

LCFAs have been shown to exert bactericidal effects on methanogens with no adaptation to the culture (Hanaki *et al.*, 1981; Koster and Cramer, 1987; Angelidaki and Ahring, 1992). Alves *et al.* (2001) showed that adaptation did occur, however, and improved the resistance of the biofilm and its biodegradation capacity with oleate. Reducing the inhibition by LCFAs was reported by adding calcium (Hanaki *et al.*, 1981) or bentonite (Angelidaki and Ahring, 1990). Calcium forms insoluble salts with LCFAs (precipitate) whereas bentonite flocculates LCFAs.

## **2.4 METABOLIC PATHWAYS OF DARK HYDROGEN FERMENTATION**

In mixed culture fermentation (MCF) a metabolic network results from the interaction of the various microorganisms. A representative metabolic network can be established by incorporating the most common catabolic reactions (Rodriguez *et al.*, 2006). The generally accepted metabolic network for glucose fermentation in MCF is shown in Figure 2.4 (Lee *et al.*, 2008). Glucose fermentation is characterized by branched pathways in which pyruvate is the central branching metabolite and the NADH/NAD redox-couple is the electron carrier. This

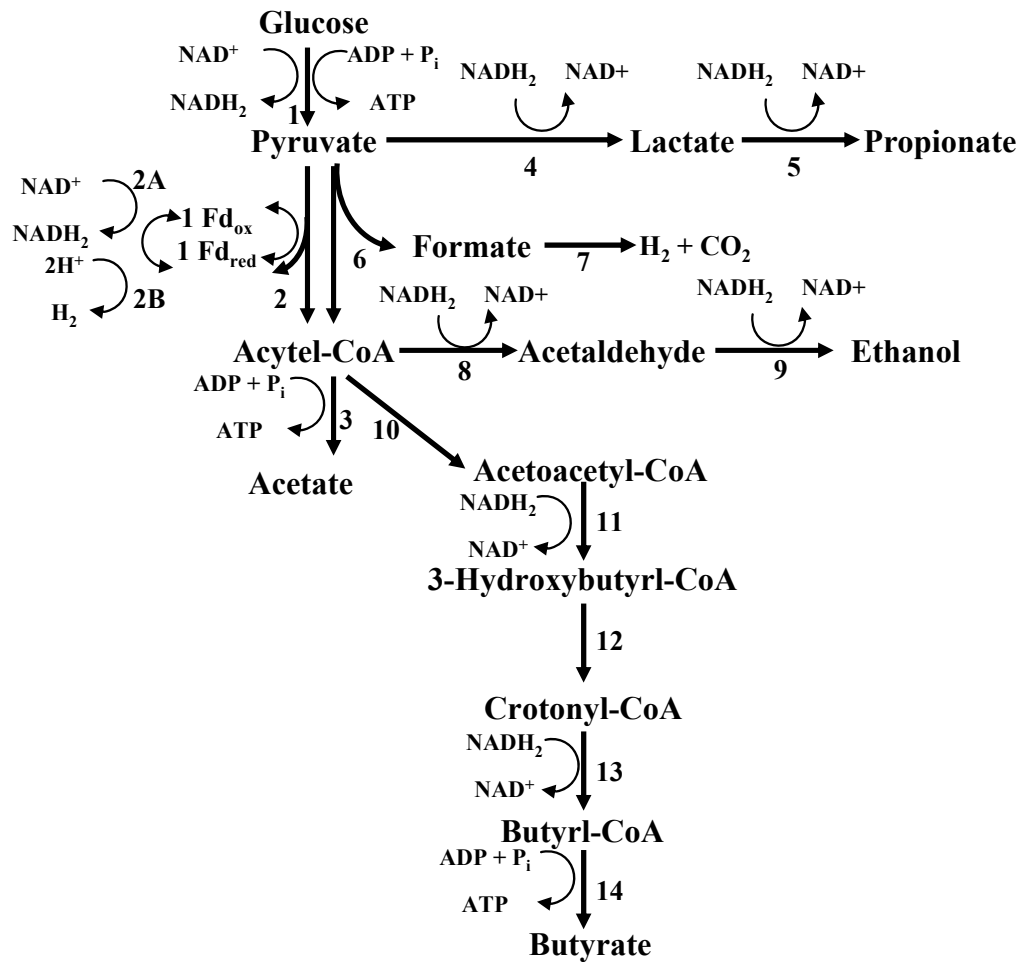


Figure 2.4 Metabolic network for anaerobic dark fermentation of glucose in mixed culture (Adapted from Lee *et al.*, 2008).

metabolic network is characterized by (Figure 2.4):

- 1- ATP synthesis and NADH generation occurs with pyruvate formation from glucose via the Embden-Meyerhoff pathway (reaction 1).  $H_2$  is produced by the oxidation of excess NADH (reaction 2).
- 2- Acetate production by pyruvate oxidation and decarboxylation with 1 ATP is formed by substrate level phosphorylation (reaction 3).
- 3- Butyrate is synthesized by decarboxylation of pyruvate with  $NAD^+$  production and propionate is produced by the reduction of pyruvate with the formation of  $1/3$  ATP in each reaction. Lactate is also produced by the reduction of pyruvate while ethanol is produced by the reduction and

decarboxylation of pyruvate, but no energy is conserved as ATP in lactate and ethanol production.

Energy conservation occurs only via substrate-level phosphorylation. ATP is produced during the initial step of glycolysis and in the reactions that ferment glucose to acetate, butyrate, and propionate (reactions 3, 14, and 5, respectively). Reducing power is generated by microorganisms in the form of intracellular electron carrier  $\text{NADH}_2$ , during glycolysis (glucose  $\rightarrow$  pyruvate  $\rightarrow$  Acetyl-CoA).

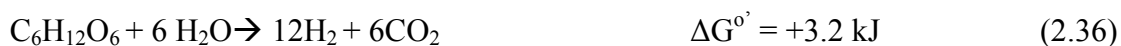
Hydrogen production occurs in two catabolic steps. 1- cleavage (decarboxylation) of pyruvate to acetyl-CoA,  $\text{CO}_2$ , and  $\text{H}_2$  (reaction 2) which is catalyzed by pyruvate:ferredoxin oxidoreductase (Chen *et al.*, 2006; Lee *et al.*, 2008) and hydrogenase (Chen *et al.*, 2006). Generation of  $\text{NADH}_2$  (reaction 2A) competes for electrons with the production of  $\text{H}_2$  through ferredoxin reduction (reaction 2B). Generation of either  $\text{NADH}_2$  or  $\text{H}_2$  eliminates the other. 2- cleavage of formate (reaction 7) which is catalyzed by formate hydrogenlyase (Lee *et al.*, 2008).

The competition between  $\text{NAD}^+$  and  $\text{H}^+$  for  $\text{Fd}_{\text{red}}$  (reaction 2) controls the  $\text{H}_2$  yield (Lee *et al.*, 2008). Coupling  $\text{Fd}_{\text{red}}$  and  $\text{NAD}^+$  generates  $\text{NADH}_2$  which is required for biomass synthesis and drives the reactions which consume  $\text{NADH}_2$  such as lactate, propionate, acetaldehyde, ethanol, and butyrate formation. Thus, the production of lactate and propionate (reactions 4 and 5) decreases the  $\text{H}_2$  yield.

Theoretically, 12 mol of  $\text{H}_2$  can be produced by the complete oxidation of glucose to  $\text{H}_2$  and  $\text{CO}_2$  (Eq. 2.36). However, 4 mol of  $\text{H}_2 \cdot \text{mol}^{-1}$  glucose is the maximum amount that is biologically possible if acetate is the only by-product with  $\text{H}_2$  (Eq. 2.1). The yield decreases to 2 mol  $\text{H}_2 \cdot \text{mol}^{-1}$  glucose when butyrate is the by-product (Eq. 2.2) instead of acetate. Hence, it was suggested that  $\text{H}_2$  production from glucose



depends on the butyrate/acetate ratio (Reith *et al.*, 2003). The H<sub>2</sub> yield depends on the fermentation pathway and end-products (Levin *et al.*, 2004).



Practically, high H<sub>2</sub> yield is found with a mixture of acetate and butyrate fermentation products, and low H<sub>2</sub> yields are associated with propionate and reduced end-products such as alcohols and lactic acid (Levin *et al.*, 2004).

The dominant pathway depends on the type of organism, oxidation state and concentration of the substrate, and environmental conditions (pH and  $P_{\text{H}_2}$ ) (Rodriguez *et al.*, 2006; Hallenbeck and Ghosh, 2009). Strict anaerobes such as *Clostridia* use reaction 2 to generate reduced ferredoxin (Fd<sub>red</sub>) which serves as electron donor for proton reduction to form H<sub>2</sub> gas. Facultative anaerobes, such as *Enterobacter* and *Klebsilla* are able to use the cleavage of formate (reaction 7) to generate H<sub>2</sub> (Lee *et al.*, 2008). These species use NADH<sub>2</sub> as a reductant to produce 2,3-butanediol, ethanol, and lactate from pyruvate (Chen *et al.*, 2006). Microorganisms may use different pathways to degrade the same or different substrates depending on the environmental conditions. For example, *Bacteroides xyloxyticus* ferments glucose via glycolysis and xylose via the pentose phosphate pathway in conjunction with glycolysis (Biesterveld *et al.*, 1994). *Clostridial* fermentation is a typical branched fermentative pathway (Kim and Gadd, 2008).

pH is an important stressing factor which influences the pathway (Zoetemeyer *et al.*, 1982). The production pathway for volatile fatty acids (VFAs) is favored at high pH, whereas the production pathway for alcohol (ethanol) is favored at low pH (<5) (Ren *et al.*, 1997; Horiuchi *et al.*, 1999).

Hydrogen partial pressure ( $P_{H_2}$ ) has a direct impact on the oxidation state of the NADH/NAD redox couple and the thermodynamic state of the various product formation pathways (Mosey, 1983). The pathway producing acetate and  $H_2$  is favored at low  $P_{H_2}$ , whereas butyrate, propionate and more reduced by-products dominate at high  $P_{H_2}$  (Thauer *et al.*, 1977; Schink, 1997). Low  $P_{H_2}$  (<10 Pa) allows the redox coenzyme NADH to release electrons as molecular  $H_2$ . More acetate, carbon dioxide, and  $H_2$  are formed rather than ethanol or butyrate; this allows additional ATP synthesis from substrate level phosphorylation (Schink, 1997).

## 2.5 CULTURE PRETREATMENT

In dark fermentation,  $H_2$  is produced by acidogens and acetogens, and consumed by hydrogenotrophic methanogens, homoacetogens, and sulfate-reducing bacteria. Higher  $H_2$  yield requires pretreatment which favors  $H_2$  producers over  $H_2$  consumers. Conflicting conclusions have been made regarding the effectiveness of various pretreatments on anaerobic mixed cultures.

The pretreatments utilized include acute physical and chemical stresses such as heat-shock (thermal) treatment (BenYi and JunXin, 2009), sterilization, microwave treatment, ultrasonication (Wang *et al.*, 2003), acid and base treatment (Chen *et al.*, 2002; BenYi and JunXin, 2009; Cai *et al.*, 2004), chemical supplementation, methanogen inhibitors (e.g. 2-bromoethanesulfonic acid (BESA), chloroform, iodopropane, acetylene, LCFAs, etc.), freeze-thawing (Wang *et al.*, 2003; Iyer *et al.*, 2004; Cheong and Hansen, 2006), repeated-aeration (Ren *et al.*, 2008), load-shock (O-Thong *et al.*, 2009), chemical acidification with and without preacidification of the sludge (Iyer *et al.*, 2004), and application of a low voltage (3.0-4.5 V) electrical current (Roychowdhury, 2000). Many of these pretreatment methods were reported to

be “the best” in comparison to other methods: e.g. treatment with acid (Cheong and Hansen, 2006), base (Zhu and Beland, 2006), BESA (Mohan *et al.*, 2008b), heat-shock (Mu *et al.*, 2007; Kotay and Das, 2009), repeated-aeration (Ren *et al.*, 2008), combinations of acid, heat and chemicals (Mohan *et al.*, 2008a, 2008b, 2007), and LCFA (Chowdhury *et al.*, 2007). The following sections present a review on the effectiveness and deficiencies of specific pretreatments on H<sub>2</sub> production.

### 2.5.1 Heat Treatment

Heat-treatment effectively selects spore-forming bacteria such as *Clostridium* (Lay *et al.*, 1999) and eliminates methanogens (Mohan *et al.*, 2008b), but it does not select exclusively for H<sub>2</sub> producing bacteria (Kraemer and Bagley, 2007). Many H<sub>2</sub> consuming bacteria are spore formers and survive the heat treatment (Table 2.6) whereas non-spore forming H<sub>2</sub> producing bacteria such as *Entrobacter* sp. (Nakashimada *et al.*, 2002) and *Citrobacter* Spp. (Oh *et al.*, 2003) are killed by heat treatment.

Table 2.6 Spore forming H<sub>2</sub> consuming bacteria.

	Microorganism	Reference
Acetogenic bacteria	<i>Acetobacterium</i>	Drake, 1994
	<i>Clostridium scatologenes</i>	Kim <i>et al.</i> , 2006
	<i>Sporomusa</i>	Drake, 1994
	<i>Clostridium aceticum</i>	Cheong and Hansen, 2006
	<i>Clostridium autotrophicum</i>	Cheong and Hansen, 2006
Propionate producer	<i>Propionibacterium</i>	Verhulst <i>et al.</i> , 1985
Lactate producer	<i>Sporolactobacillus</i>	Kim and Gadd, 2008
Sulphate-reducers	<i>Desulfotomaculum</i>	Madigan <i>et al.</i> , 2000

Researchers have applied heat treatment by means of wet and dry heat, desiccation, boiling, and oven-drying at various temperatures. Oven drying at 105°C for 2 h was used to kill off vegetative bacteria (Logan *et al.*, 2002; Iyer *et al.*, 2004). Some of non-spore forming bacteria which could consume H<sub>2</sub> or lower its production such as

*Bacteroides* and *Lactobacillus* can survive dry heat treatment (Iyer *et al.*, 2004; Ahn *et al.*, 2005; Kim *et al.*, 2006). Moreover, according to Duangmanee *et al.* (2007), repeated heat-treatments were necessary when the H<sub>2</sub> production declined after one month of continuous operation.

Baghchehsaraee *et al.* (2008) reported H<sub>2</sub> yield of 2.3 mol·mol<sup>-1</sup> glucose from heat-treated (65 °C for 30 min) anaerobic-digested mixed culture in a batch reactor. These researchers observed a decrease in the H<sub>2</sub> yield and a decrease in the microbial diversity when the temperature of the heat treatment was increased from 65 to 90 °C. Mohan *et al.* (2008b) reported that heat treatment of 100 °C for 1 h resulted in relatively low H<sub>2</sub> yield. Heat (80 °C) applied for 3 hrs to 3% total solids (TS) established inocula rich in H<sub>2</sub>-producing microorganisms, but the time required for optimum heat treatment increased with the percentage of the total solids (Valdez-Vazquez and Poggi-Varaldo, 2009).

Generally, heat pretreatment alone was either not sufficient or inconsistent in developing a suitable H<sub>2</sub>-producing microbial consortium (Kotay and Das, 2009) because it does not exclusively select these microorganisms (Kraemer and Bagley, 2007). Moreover, heat pretreatment does not remove methanogens completely and they resume their growth when the conditions are favorable (Wang *et al.*, 2007; Oh *et al.*, 2003).

### **2.5.2 Acid/base Treatment**

Several reports have used acid (pH 3) or base treatment (pH 9 to 11.5) as a means of increasing H<sub>2</sub> production in mixed cultures (Chen *et al.*, 2002; Zhu and Beland, 2006; Mohan *et al.*, 2008b; BenYi and JunXin, 2009; Cai *et al.*, 2004; O-Thong *et al.*, 2009). Acidifying a mixed culture at pH 3 with HCl for 30 min repressed both methanogens and H<sub>2</sub>-producers. However, increasing the pH to 10 with NaOH for 30

min did not repress methanogens completely and significantly influenced  $H_2$  production (Zhu and Beland, 2006). After one treatment regimen, the yield reached  $1.44 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ sucrose}$  and increased to  $6.12 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ sucrose}$  after treating the culture twice with NaOH (Zhu and Beland, 2006). The duration of the treatment with acid or base was significant factor according to Chen *et al.* (2002). Chen *et al.* (2002) also reported increasing  $H_2$  yields in a sewage culture (333 and 200 times) after increasing the acid or base treatment duration to 24 h (Chen *et al.*, 2002).

### 2.5.3 Methanogen Inhibitors

Many reports have provided evidence describing oxygen, BESA, acetylene, ethylene, ethane, methyl chloride, methyl fluoride, lumazine, chloroform, iodopropane, and LCFAs as methanogenic inhibitors (Valdez-Vazquez and Poggi-Varaldo, 2009). For example, flushing an anaerobic mixed culture with oxygen for 30 min repressed the methanogens completely (Zhu and Beland, 2006; Ueno *et al.*, 1995, 1996). BESA is a specific methanogenic inhibitor (Sparling and Daniels, 1987; Sparling *et al.*, 1997). This chemical behaves as a structural analog of coenzyme M (CoM) in methanogens (DiMarco *et al.*, 1990). BESA inhibits the reductive demethylation of methyl-S-CoM in the last step of methanogenesis (Muller *et al.*, 1993). Concentrations of 25 mM (Sparling *et al.*, 1997) and 100 mM (Wang *et al.*, 2003) have been shown to improve  $H_2$  production. However, application of a lower concentration (10 mM) BESA with iodopropane for 30 min inhibited methanogens but did not improve  $H_2$  production (Zhu and Beland, 2006). Cost, toxicity, and reports of spontaneous resistance in some methanogens (Smith and Mah, 1981) are issues limiting the applicability of BESA in industrial scale  $H_2$  production.

Acetylene is a known methanogenic inhibitor (Sprott *et al.*, 1982); however, it has been shown not to affect the  $H_2$  yield and production rate in *C. thermocellum* because

it does not interfere with hydrogenase activity (Valdez-Vazquez and Poggi-Varaldo, 2009). After sparging with nitrogen, inhibition of methanogens by acetylene is not reversible (Oremland and Taylor, 1975). Acetylene is as effective as chloroform and BESA in improving H<sub>2</sub> production in anaerobic digestion according to Sparling *et al.*, (1997), therefore, it was suggested as an excellent candidate for large-scale industrial H<sub>2</sub> production (Valdez-Vazquez and Poggi-Varaldo, 2009).

Chloroform inhibits methanogenic activity because it blocks the function of corrinoid enzymes and inhibits methyl-coenzyme M reductase (Chidthaisong and Conrad, 2000). Thus, it was thought to enhance the H<sub>2</sub> yield (Liang *et al.*, 2002; Cheng *et al.*, 2003). Chloroform inhibited methanogen granules more than acid treatment (pH 3 for 24 hrs) and improved H<sub>2</sub> production at low concentrations 0.05%; nevertheless, 2.5% chloroform severely inhibited H<sub>2</sub> production (Hu and Chen, 2008). Abreu *et al.* (2008) found that although combined BESA/chloroform treatment reduced homoacetogenesis, 30 uM chloroform for 72 hrs inhibited some H<sub>2</sub>-producing bacteria and decreased the yield of hydrogen.

With so many options available for the inhibition of methanogens, selection of an inhibitor depends on factors such as capital cost of a chemical feed system, operational costs, environmental friendliness, chemical toxicity, technical feasibility and complexity, effectiveness of the inhibition, stabilization times of the inoculum, friendliness to H<sub>2</sub>-producing microorganisms, inocula origin and substrate type (Valdez-Vazquez and Poggi-Varaldo, 2009).

#### **2.5.4 LCFA in Hydrogen Production Studies**

LCFAs are known inhibitors of methanogens (Koster and Creamer, 1987; Lalman and Bagley, 2001 and 2002). In section 2.3.6, the toxicity of LCFAs to methanogens and other microorganisms was described. Thus, LCFAs have been investigated in H<sub>2</sub>

production studies as alternative, environmentally friendly, and biodegradable inhibitors. In batch mixed culture, 2,000 mg L<sup>-1</sup> of C18:2 and C18:1 enhanced the yield of H<sub>2</sub> during glucose fermentation. A H<sub>2</sub> yield of 2.37 mol H<sub>2</sub>·mol<sup>-1</sup> glucose was reported by Chowdhury (2007) from mixed culture incubated at 37°C with an initial pH of 5.0 and fed 2,000 mg L<sup>-1</sup> linoleic acid (LA). At pH 7.6, the same author obtained a yield of 1.55 mol H<sub>2</sub>·mol<sup>-1</sup> glucose. When LA was replaced with oleic acid (OA), the yields were 2.14 and 1.44 mol H<sub>2</sub>·mol<sup>-1</sup> glucose for pH 5.0 and 7.6, respectively. Hydrogen yields of 0.77 and 1.27 mol H<sub>2</sub>·mol<sup>-1</sup> glucose were obtained from mixed culture incubated at 25°C with an initial pH of 7.6 and fed 2,000 mg L<sup>-1</sup> LA and OA, respectively and lowering the pH to 5.0 increased the yield to 2.7 and 2.2 mol H<sub>2</sub>·mol<sup>-1</sup> glucose, respectively (Gurukar, 2005).

### **2.5.5 Summary of literature on pretreatments**

Several studies investigated several pretreatments comparatively on the same culture. Zhu and Beland, (2006) compared the effects of heat-shock, acid, base, aeration, BESA, and iodopropene on H<sub>2</sub> yield from sucrose. They found that the yields varied by a factor of four, and iodopropene was the best and base treatment was the worst. Mohan *et al.* (2008b) found that acid treatment (pH 3.0 for 24 hrs) was the least effective in improving H<sub>2</sub> production in comparison to chemical treatment (BESA), heat treatment, and different combinations of these three methods. BenYi and JunXin (2009) found that the H<sub>2</sub> yield from alkaline (pH 11.5) pretreated sewage culture was higher than from heat treatment.

Favourable pretreatment alters the microbial community structure by enhancing the H<sub>2</sub> producing population (O-Thong *et al.*, 2009). Different pretreatments are known to establish different microbial communities (Ren *et al.*, 2008). For example, *Clostridium* sp. dominated heat and acid-treated cultures while *Bacillus* sp. dominated

BESA-treated cultures (O-Thong *et al.*, 2009). The metabolic pathways, fermentation type, and distribution of by-products are functions of the type of microorganisms (Hallenbeck and Ghosh, 2009). However, the specific effects of each pretreatment on the activity and population of the various trophic groups of the mixed culture have not been quantified in any study.

In conclusion, comparative studies collectively confirm that no specific pretreatment is the best to select for maximizing H<sub>2</sub> yields. Moreover, individual pretreatments alone are not sufficient to increase the H<sub>2</sub> yield.

## **2.6 ROLE OF pH IN HYDROGEN PRODUCTION AND CONSUMPTION**

pH is an important operational and stress factor during fermentative H<sub>2</sub> production. It is the most extensively studied parameter because of its impact on H<sub>2</sub>-producing and H<sub>2</sub>-consuming microorganisms. Within the range in which microorganisms grow, pH influences the utilization of carbon and energy sources, efficiency of substrate degradation, synthesis of proteins and storage material, and release of metabolic products (Baily and Ollis, 1986). Gottschalk (1986) concluded that pH variation can affect morphology and cell structure, and consequently flocculation and adhesion phenomena. The activity of several enzymes of *C. tyrobutyricum* are pH dependent; increased activity of phosphotransacetylase (PTA) and lactate dehydrogenase (LDH) was found at pH 5.0 (Zhu and Yang, 2004). These enzymes control the formation of acetate and conversion of pyruvate to lactate in *C. tyrobutyricum*.

pH controls the metabolic pathway and the activity of hydrogenase during fermentative H<sub>2</sub> production (Dabrock *et al.*, 1992;). Changes in pH can affect the fermentation pathway used by bacteria, alter the distribution of the by-products (Zoetemeyer *et al.*, 1982), and directly influence H<sub>2</sub> production. The relative amounts



of the main VFAs are strongly dependent on pH and pH 4.0 to 5.0 favors the production of propionate and H<sub>2</sub> while pH 6.0 to 7.0 promotes the formation of acetate, butyrate, and i-butyrate, with a transition zone between pH 5.0 and 6.0 (Zoetemeyer *et al.*, 1982). Inocula adapted to neutral conditions channel reducing equivalents to produce formate when they are pH shocked, whereas acid-adapted inocula (pH 5.0-6.5) produced more H<sub>2</sub> (Voollapalli and Stucky, 2001). *Clostridia*, for example, produce acid at medium pH and switch to solvent production at low pH (Wiegel *et al.*, 2006). Generally, Voollapalli and Stucky (2001) found that H<sub>2</sub> production was influenced mainly by the pH of the source of the inocula.

Many studies have examined the effect of pH on H<sub>2</sub> fermentation from glucose and sucrose using mixed cultures (Tables 2.3, 2.4 and 2.7). Wang *et al.* (2007) found that  $P_{H_2}$  in biogas, H<sub>2</sub> production rate, and H<sub>2</sub> yield were pH-dependent in two inocula. Initial pH can also affect the extent of the lag phase on H<sub>2</sub> production in batch reactors. According to Kapdan and Kargi (2006), a low initial pH of 4.0 to 4.5 caused long lag phase periods of 20 h; however, acid treatment of the inoculum (pH 3.0, 18 h) shortened the lag phase (Cheng *et al.*, 2002). An optimum initial pH between 5.0 and 6.5 has been reported for H<sub>2</sub> production (Li and Fang, 2007; Van Ginkel *et al.*, 2001).

Kawagoshi *et al.* (2005) suggested that a constant pH of 6.0 is the most suitable to ferment organic waste material to H<sub>2</sub> using mixed bacterial cultures. A change of 0.5 units from the optimal pH decreased the H<sub>2</sub> production efficiency by 20% (Fang and Liu, 2002; Oh *et al.*, 2003; Lin and Cheng, 2006; Lin *et al.*, 2006; Mu *et al.*, 2006; Valdez-Vazquez and Poggi-Varaldo, 2009). Therefore, Valdez-Vazquez and Poggi-Varaldo (2009), concluded that the optimal pH must be determined for each inoculum because pH control is crucial to H<sub>2</sub> production.

Table 2.7 Effect of pH on H<sub>2</sub> yield from glucose fermentation.

Incoula	Type of reactor	pH	Temp (°C)	H <sub>2</sub> Yield (mol·mol <sup>-1</sup> )*	Reference
SS	CSTR	5.7	35	1.7	Lin and Chang, 1999
ADS	CSTR	5.5	36	2.1	Fang and Liu, 2002
Soybean meal	CSTR	6.0	36	1.44	Mizuno <i>et al.</i> , 2000b
ADS	CSTR	5.5	36	1.91	Fang <i>et al.</i> , 2002b
ADS	UASB	4.0	35	1.51	Wang <i>et al.</i> , 2007
SS	CSTR	5.5	36	1.91	Fang <i>et al.</i> , 2004
SS	Sequencing continuous reactor	5.0	35	1.47	Hwang <i>et al.</i> , 2004
Soil	CSTR	5.5	30-37	1.80	Iyer <i>et al.</i> , 2004
SS	CSTR	6.2	15-34	1.42	Lin and Chang, 2004
Acclimated sludge	Trickling biofilter	5.5	55-64	1.11	Oh <i>et al.</i> , 2004
ADS	Batch	6.2	25	0.97	Oh <i>et al.</i> , 2003
ADS	batch	5.7	35.5	0.8-1.0	Cheong and Hansen, 2006
SS	Batch	5	35	0.84	Chen <i>et al.</i> , 2002
Soil	Batch	6.0	26	0.92	Logan <i>et al.</i> , 2002
ADS	Batch	6.5	37	1.07	Cubillos <i>et al.</i> , 2010

\* Calculated as 1 ml H<sub>2</sub> per g = 0.000736 mol H<sub>2</sub> · mol<sup>-1</sup> glucose. ADS = anaerobic digested sludge; SS = sewage sludge.

The undissociated acid concentration is pH-dependent, and is greater when pH < pK<sub>a</sub> (Valdez-Vazquez and Poggi-Varaldo, 2009). The pK<sub>a</sub> values of acetate, butyrate and lactate are 4.82, 4.75 and 3.86, respectively (Kim and Gadd, 2008). Production of these acids by carbohydrate fermenting bacteria decreases the pH values to near or lower than the pK<sub>a</sub> and undissociated forms of the acids accumulate. The undissociated acids are toxic because they are hydrophobic and they are able to cross the cytoplasmic membrane and dissipate the proton motive force (Kim and Gadd, 2008).

The internal pH (pH<sub>i</sub>) of the cell depends on the external pH of the medium. Microorganisms producing weak acids are unable to maintain a constant pH<sub>i</sub> (Menzel and Gottschalk 1985). At extracellular pH <4.7, undissociated acids cross the cytoplasmic membrane freely, accumulate inside the cell at large ΔpH values and

subsequently decrease the  $pH_i$  (Russell and Diez-onzalez, 1997). The high concentrations of low molecular-weight VFAs produced during acidogenesis collapse the pH gradient across the membrane and inhibit all metabolic functions in the cell (Gottschalk, 1986).

The growth rates of the trophic groups in anaerobic cultures can change with pH. The pH range for optimal growth is known to differ for each group. For example, acidogens grow optimally at pH 5.9 (Horiuchi *et al.*, 1999) whereas acetogens, hydrogenotrophic and acetoclastic methanogens grow optimally at an approximate pH of 7.0 (Gujer and Zehnder, 1983). pH changes may cause drastic shifts in the relative numbers of different species in a heterogeneous population (Horiuchi *et al.*, 1999). Low pH levels ( $< 4.0$ ) can inhibit the activity of all microorganisms in a  $H_2$  producing culture (Hwang *et al.*, 2004) while no  $H_2$  was produced from sucrose at pH 4.0 (Zhang *et al.*, 2003).

pH levels of approximately 6.6 can decrease the growth rate of both acetoclastic and hydrogenotrophic methanogens during the anaerobic conversion of carbohydrates (Mah *et al.*, 1977). Low pH can limit  $H_2$  metabolism by consumers such as hydrogenotrophic methanogens and sulfate reducers (Mah *et al.*, 1977). Goodwin *et al.* (1998) reported that the  $H_2$  turnover rate ( $k_a$ ) and the maximum  $H_2$  uptake velocity ( $V_{max}$ ) were direct functions of pH. Nevertheless, half-saturation concentration ( $K_s$ ) was independent of pH. Surprisingly, some studies have shown methanogenesis can proceed at low pH in continuous  $H_2$  production (Hwang *et al.*, 2004; Speece, 1996).

Operating under low pH conditions can inhibit methanogenic activity and subsequently produce an inoculum rich in  $H_2$ -producers. The main limitations of this method are acclimatization and the eventual presence of acetogenic and methanogenic  $H_2$ -consuming microorganisms. An increasing number of reports have provided

evidence showing the occurrence of methanogenesis under low pH conditions (Hamberger *et al.*, 2008; Castelloa *et al.*, 2009; Jain and Mattiasson, 1998). Many reports have shown acidophilic or acidotolerant methanogenesis (Brauer *et al.*, 2006; Florencio *et al.*, 1993; Goodwin and Zeikus, 1987; Sizova *et al.*, 2003; Williams and Crawford, 1985). *Methanosarcina* and *Methanobacterium* can grow and produce methane at pH values as low as 5.0 to 4.68 (Maestrojuan and Boone, 1991; Patel *et al.*, 1990; Taconi *et al.*, 2007). Kotsyurbenko *et al.* (2007) have also reported strongly increased  $H_2$ -dependent methanogenesis at very low pH (3.8).

The optimum pH for LCFAs inoculated anaerobic culture is approximately 5.0. A yield of  $2.7 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose was reported from mesophilic culture fed 5,000 mg  $L^{-1}$  glucose plus 2,000 mg  $L^{-1}$  linoleic acid with an initial pH 5.0; however, a yield of  $2.2 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose was obtained when oleic acid was used instead of linoleic acid under the same conditions (Chowdhury, 2007). When the initial pH was increased to 7.6 lower yields of 1.11 and  $1.27 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose were reported at room temperature from a feed consisting of glucose plus linoleic or glucose plus oleic acids, respectively (Gurukar, 2005).

## 2.7 HYDROGEN YIELD IMPROVING TECHNIQUES

Mixed culture fermentation could produce  $H_2$  up to the maximum theoretical limit ( $4 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose); however,  $H_2$  is consumed by fermentative bacteria, hydrogenotrophic methanogens, homoacetogens, and sulfate reducing bacteria. Fermentative bacteria consume  $H_2$  when they produce lactate, propionate, butyrate, and alcohols (Eq. 2.4, 2.5, and Figure 2.4). Thus, controlling and minimizing  $H_2$  consumption is crucial to increase the molar yield.

Techniques that minimize  $H_2$  consumption and increase the molar yield include sparging with inert gas (Mizuno *et al.*, 2000a), increased stirring (Lamed *et al.*, 1988),

decreasing the headspace pressure in the reactor (Park *et al.*, 2005), removing dissolved gases by immersed membrane (Liang *et al.*, 2002), and optimizing the organic loading rate (Kim *et al.*, 2006).

Sparging with an inert gas (N<sub>2</sub>, CO<sub>2</sub>, or Argon) can increase the molar yield of H<sub>2</sub> (Mizuno *et al.*, 2000a; Van Groenestijn *et al.*, 2002; Oh *et al.*, 2002; Hussy *et al.*, 2003; Kim *et al.*, 2006). For example, 20 to 70% improvement in the H<sub>2</sub> yield was reported for N<sub>2</sub> sparging, 80-120% for CO<sub>2</sub>, and 88% for methane, whereas sparging with H<sub>2</sub>/CO<sub>2</sub> resulted in only 0-12% improvement (Kraemer and Bagley, 2007). Sparging can also affect the by-products distribution (Tanisho *et al.*, 1998; Crabbendam *et al.*, 1985). Van Groenestijn *et al.* (2002) stripped H<sub>2</sub> from the reactor with steam to maintain low H<sub>2</sub> and carbon dioxide partial pressures. According to Van Groenestijn *et al.* (2002), the energy required to run this process is at least four times lower than the combustion value of the H<sub>2</sub> gas produced in such reactors.

Removal of dissolved gases is known to limit homoacetogenesis (Hussy *et al.*, 2003); however, to date no clear experimental evidence has been published. Organic loading rate (OLR) can maximize the H<sub>2</sub> yield; however, conflicting results have been reported in several studies (Kim *et al.*, 2006; Wu *et al.*, 2006; Yang *et al.*, 2006). In conclusion, the role and activity of different microorganisms responsible for H<sub>2</sub> consumption are not well defined and quantified. For these reasons, the following sections present a review on the dynamics and kinetics of the various trophic groups associated with mixed anaerobic cultures.

## **2.8 CULTURE DYNAMIC AND KINETICS**

The growth of mixed cultures is complicated because the state variables of the system change with time and with respect to each other. Mixed culture has the potential to produce H<sub>2</sub> efficiently up to the maximum theoretical levels (Kotay and

Das, 2009). The successful application of mixed cultures to H<sub>2</sub> production requires an operational understanding of the functional bacterial populations (Harper and Pohland, 1986) under different adopted stress conditions. Such an understanding includes substrate conversion rates and the influence of intermediates as inhibitors or stimulants. Kinetics data provide the basis for process analysis, control, and design (Pavlostathis and Giraldo-Gomez, 1991). Various trophic groups degrading reduced organic substrate in anaerobic environments vary in their optimal growth conditions. Thus, they differ in terms of their activity and abundance.

### **2.8.1 Activity of anaerobic trophic groups**

Understanding the kinetics of anaerobic H<sub>2</sub> production systems is essential in order to develop them to their fullest potential, yet such understanding is complicated by the complex nature of mixed microbial populations. The composition of mixed culture consortia includes acidogenic, proton-reducing acetogenic, homoacetogenic bacteria, hydrogenotrophic methanogens, and aceticlastic methanogens (Demirel and Scherer, 2008). The activity and the performance of these major trophic groups during fermentative H<sub>2</sub> production are still not fully defined. Homoacetogenic and acetogenic bacteria have received less attention (Dolfing, 1988; Schink, 1994) than acidogens and methanogens, especially in H<sub>2</sub> production studies (Abreu *et al.*, 2007). Both qualitative and quantitative information about the microbial population dynamics and the activities of the various trophic groups are required to design efficient fermentative H<sub>2</sub> production systems. Better design of a H<sub>2</sub> production system would increase the substrate's degradation capacity, production rate, and yield.

Based on bioenergetic analyses of the anaerobic conversion process, Pavlostathis and Giraldo-Gomez (1991) estimated the specific substrate utilization rate for carbohydrates, propionate, butyrate, acetate and H<sub>2</sub> to be 15.9, 8.4, 8.7, 8.4, and 29.2

(g COD g<sup>-1</sup> VSS d<sup>-1</sup>), respectively. The specific substrate utilization rates of carbohydrates and H<sub>2</sub> are generally higher than rates for VFAs. The minimum doubling time of VFAs degrading microorganisms is one order of magnitude higher than carbohydrate degrading microorganisms (Pavlostathis and Giraldo-Gomez, 1991).

The maximum specific substrate utilization rates reported so far for the various trophic groups in anaerobic fermentation are summarized in Table 2.8 (A), (B) and (C) which indicate that the activity varies under different conditions. Reported maximum substrate utilization rates for glucose and its by-products in anaerobic digestion vary widely. For example, the maximum glucose specific utilization rate ranged from 0.4 d<sup>-1</sup> to 179 d<sup>-1</sup> (Table 2.8 (A)) (Lettinga, 1995) while for lactate the range is between 0.24 to 24.9 d<sup>-1</sup> (Table 2.8 (A)).

The activity of H<sub>2</sub> consuming microorganisms has been reported to be as high as 39.1 d<sup>-1</sup> (Shizas *et al.*, 2001) and as low as 0.00864 d<sup>-1</sup> (Table 2.8 (C)) (Thiele *et al.*, 1988). Hydrogen maximum specific utilization rate data reported showed not only methanogenic but also acetogenic H<sub>2</sub> consumption. Generally, large values of H<sub>2</sub> consuming activity have been reported for mixed cultures (Table 2.8 (C)). Because no standard laboratory test is agreed upon for measuring the activity of the biomass, each laboratory adopts its own procedure and this might have contributed to the large variation in the reported values. The maximum specific utilization rates for acetate, propionate, and butyrate are highly variable. Noticeably, flocculated cultures had lower activities for degrading VFAs than granulated cultures (Table 2.8 (B)).

Table 2.8 (A) Maximum glucose and lactate specific utilization rates in anaerobic cultures.

Substrate	Culture	$ka$ (day) <sup>-1</sup>	Reference
Glucose	Methane phase CSTR (37°C)	3.4	Pohland and Ghosh, 1971
	Continuous (35°C)	70.6	Noike <i>et al.</i> , 1985
	Mixed culture mesophilic anaerobic digestion	1.33-70.6	Pavlostathis and Giraldo-Gomez, 1991
	Acid producing sludge	9.5	Lettinga, 1995
	Mixed culture	2.5	
	Mixed culture	179	
	Dispersed culture CSTR	63	
	Dispersed culture UASB HRT<81 min	24-33	
	Dispersed culture UASB HRT>26 min	11-19	
	Aggregates gaslift	74	
	Granular sludge 1 step UASB	0.4	
	Granular sludge 2 step UASB,	1.6-2.2	
	Acidogenic reactor	62.21	Dolfing <i>et al.</i> , 1985
	Acidogens floc (35°C)	8.11	Krylow and TalFigiel, 2004
	Acidogens granules (35°C)	8.45	
	Anaerobic sludge (30°C)	51	Gujer and Zehnder, 1983
	Mixed culture (35-37°C)	125	Pavlostathis and Giraldo-Gomez, 1991
	Mixed anaerobic cultures from dairy, olive-mill, piggery, and glucose-fed digesters	25.92	Gavala and Lyberatos, 2001
	anaerobic sludge for food waste treatment (35°C)	3.58	Ortega <i>et al.</i> , 2008
	Domestic wastewater anaerobic sludge (21°C)	19.3	Shizas <i>et al.</i> , 2001
Lactate	Mixed anaerobic cultures from dairy, olive-mill, piggery, and glucose-fed digesters	0.24	Gavala and Lyberatos, 2001
	Domestic wastewater anaerobic sludge (21°C)	24.9	Shizas <i>et al.</i> , 2001



Table 2.8 (B) Maximum acetate, propionate, and butyrate specific utilization rates in anaerobic cultures.

Substrate	Culture	$ka$ (day) <sup>-1</sup>	Reference
Acetate	Continuous mixed culture (35°C)	8.7	Lawrence and McCarty, 1969
	Batch mixed culture, (20°C) (35°C)	2.6 2.6-5.1	Van den Berg, 1977
	Continuous acetate enrichment, (35°C)	8.5	Kugelman and Chin, 1971
	Continuous mixed culture (35°C)	4.4-11.6	Noike <i>et al.</i> , 1985
	Mixed culture mesophilic anaerobic digestion	2.6-11.6	Pavlostathis and Giraldo-Gomez, 1991
	Digester sludge	8.3	Middleton and Lawrence, 1977
	Mixed culture CSTR	8.5	Lettinga, 1995
	Granular sludge UASB	3.1	
	Methanogens - granules (35°C)	1.68	
	Methanogens - flocs (35°C)	0.76	
	Methanogens	0.07-0.21	Lay <i>et al.</i> , 1998,
	Mixed anaerobic cultures from dairy, olive-mill, piggery, and glucose-fed digesters	0.22	Gavala and Lyberatos, 2001
	anaerobic sludge for food waste treatment (35°C)	0.47	Ortega <i>et al.</i> , 2008
	Mixed anaerobic sludge (37°C)	7.9	Vavilin <i>et al.</i> , 1996
	Domestic wastewater anaerobic sludge (21°C)	7.56	Shizas <i>et al.</i> , 2001
Propionate	Continuous mixed culture: (25°C) (35°C)	7.8 7.7	Lawrence and McCarty, 1969
	Anaerobic sludge (25-35°C)	0.31	Gujer and Zehnder, 1983
	Mixed culture	3.4-19	Pavlostathis and Giraldo-Gomez, 1991
	Mixed anaerobic sludge (37°C)	19- 3	Vavilin <i>et al.</i> , 1996
	Domestic wastewater anaerobic sludge 21°C	1.6	Shizas <i>et al.</i> , 2001
	UASB (37 °C): Granules Flocs	0.67 0.53	Schmidt and Ahring, 1995
Butyrate	Continuous mixed culture (25°C)	5.0	Lawrence and McCarty, 1969
	Anaerobic sludge (35°C)	5.6	Gujer and Zehnder, 1983
	Mixed culture (35-60°C)	14	Pavlostathis and Giraldo-Gomez, 1991
	Domestic wastewater anaerobic sludge (21°C)	3.3	Shizas <i>et al.</i> , 2001
	Continuous mixed culture (25°C)	5.0	Lawrence and McCarty, 1969
	UASB (37 °C): Granules Flocs	1.13 0.74	Schmidt and Ahring, 1995

Table 2.8 (C) Maximum H<sub>2</sub> specific utilization rates in anaerobic cultures.

Culture	$ka$ (day) <sup>-1</sup>	Reference
Propionate-adapted mixed culture (35°C)	16.5	Pavlostathis and Giraldo-Gomez, 1991
Mixed culture mesophilic anaerobic digestion	1.90-90	Pavlostathis and Giraldo-Gomez, 1991
Digesting sludge	1.1	Shea <i>et al.</i> , 1968
Primary sludge (35°C)	44-178	Siegrist <i>et al.</i> , 2002
Methanogens	0.06-0.41	Lay <i>et al.</i> , 1998
Acetogens	0.07-0.09	
Anaerobic sludge for food waste treatment (35°C)	0.26	Ortega <i>et al.</i> , 2008
Free flora (35°C)	0.01	Thiele <i>et al.</i> , 1988
Flocs (35°C)	0.04	
Domestic wastewater anaerobic sludge (21°C)	39.1	Shizas <i>et al.</i> , 2001
Digester sludge (30°C)	11-69 mg COD l <sup>-1</sup> .h	Robinson and Tiedje, 1982
Batch acetogenic culture	1.97±1.12 umol.mgTSS <sup>-1</sup> .day <sup>-1</sup>	Rossetti <i>et al.</i> , 2003

## 2.8.2 Composition of the mixed culture

Mixed cultures are composed of biotic and abiotic particles. Different microbial trophic groups share different niches and are involved in anaerobic fermentation to decompose the reduced organic matter. Not all of the biotic particles are active microorganisms. The instantaneous concentration of a specific substrate depends on the active biomass that degrades them. Therefore, the ability of an anaerobic culture to treat various organic compounds depends strongly on the particular mixture of bacterial species (Gavala and Lyberatos, 2001). Defining the limiting reaction rate in H<sub>2</sub> production under different stress conditions requires quantifying the size of individual active trophic groups and their associated activities.

Traditionally, the volatile suspended solids (VSS) concentration was used as a measure to represent the amount of bacteria. In addition to VSS, various parameters and methods such as microscopic or culture-based counts (most probable number

(MPN), autofluorescence for methanogens), molecular techniques such as analysis of cloned 16S rRNA gene fragments, terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and fluorescence in situ hybridisation (FISH) have been used to determine the anaerobic microbial communities in dark H<sub>2</sub> fermentation (Curtis *et al.*, 2003; Fernandez *et al.*, 1999; Godon *et al.*, 1997). These methods do not allow precise estimation of the diversity, composition, and dynamics of microbial populations under different operational parameters (Valdez-Vazquez and Poggi-Varaldo, 2009). For example, Rezanian *et al.* (2006) reported that the steady-state biomass levels in a sequencing batch reactor was composed of 41% active biomass, 25.6% cell debris and 33.4% extracellular polymeric substance. The extracellular polymers (ECP) levels were between 0.6 to 20% of the VSS in granular sludge (Schmidt and Ahring, 1996). Therefore, the methods used previously are informative, but are not sufficient to be used as a design tool to optimize the biological H<sub>2</sub> production process (Akarsubasia *et al.*, 2005).

Review of published data reveals that the estimated proportions of the various trophic groups are variable depending on the method of estimation. Acidogens were 90% of the total population in an anaerobic digester (Zeikus, 1980) while acetogens formed more than 70% and methanogens 10-15% of the microbial community in continuously stirred tank reactors (CSTRs) (Dar *et al.*, 2008). Using FISH analysis, proportions of bacteria and methanogens to total cells were 85 and 1%, respectively, in a cattle-manure-fed anaerobic digester (Lubken *et al.*, 2007). The proportions were 90% (acidogens/acetogens) and 10% methanogens for primary anaerobic sludge (Von Munch *et al.*, 1999). Solera *et al.* (2002) estimated the methanogenic population was 0.71% in an acidogenic reactor and 26% in a methanogenic reactor with two-phase anaerobic treatment. According to Sundh *et al.* (2003), the methanogenic population

constituted 4 to 8% of the bacterial biomass based on the ratio between the phospholipid fatty acids (PLFAs; eubacteria and eucaryotes) and di-ethers (PLEL; archaea).

In a pilot scale reactor treating brewery wastewater, the methanogenic/eubacterial cell number ratio was 7-8% (Ince *et al.*, 1997), whereas in anaerobic reactors treating sludge from wastewater treatment facilities, the rRNA yielded a ratio of 5-10% (Raskin *et al.*, 1995). Thiele *et al.* (1988) indicated that less than 6% of all the CO<sub>2</sub>-dependent methanogenesis in a sewage sludge digester was dependent on the dissolved H<sub>2</sub> turnover rate. Valcke and Verstraete, (1983) reported that acetoclastic methanogens composed 9.9 to 10.3% of the VSS in cultures from a municipal wastewater digester, and 14% in piggery manure. Using different methods of estimation, the relative methanogenic biomass in biogas reactors converges to approximately 10% (Sundh *et al.*, 2003). In comparison, the hydrogenotrophic methanogens population in a packed-bed reactor was approximately 5% of the total biomass population (Harper and Pohland, 1990).

### **2.8.3 Substrate adaptation**

Bioprocess performance is primarily linked to the structure of the microbial community in bioreactors (Demirel and Scherer, 2008). Microorganisms are grown under selected conditions to enhance their growth. This approach has been used to define the effects of inocula source on H<sub>2</sub> production. However, the growth conditions and the selective enrichment applied in the laboratory impose severe limitations and selective pressures for or against most microorganisms (Angert, 1998). For example, the bacterial populations depend mainly on the growth media and maintenance procedures (Sørheim *et al.*, 1989). Nutrient-rich media in the laboratory might bias

selection towards certain species that are different from the originally dominant species in the source environment.

The adaptation of an anaerobic culture to a specific substrate induces significant changes within the microbial population. It establishes a mixture of bacterial populations that differs from the mother culture both kinetically and structurally (Labat and Garcia, 1986; LaPara *et al.*, 2006). Kinetical changes are explained by the modified ability of the mixed culture to consume various substrates such as glucose, lactic, propionic, and acetic acids (Gavala and Lyberatos, 2001). Recent molecular biology techniques have assisted in explaining the acclimation-induced structural changes (Guieysse *et al.*, 2001; LaPara *et al.*, 2006). The microbial composition of sludge depends on the type of substrate and the environmental conditions (Zheng and Raskin, 2002). Glucose addition can increase the total biomass by approximately 7% of the added glucose carbon, but the methanogenic population remained unaffected (Sundh *et al.*, 2003). The methanogenic activity of cultures fed with a mixture of acetate plus propionate decreased by 30-70% after adding 10% sucrose to the growth medium (Dolfing and Bloemen, 1985).

Acclimation of two anaerobic mixed cultures from the same origin for 18 months to lactose and gelatin affected the stoichiometry of VFAs produced, and changed the maximum specific utilization rates of various compounds (Gavala and Lyberatos, 2001). Bacterial decay rates and the maintenance energy requirements increase when toxic compounds were co-metabolized (Criddle, 1993; Strydom *et al.*, 2001). Biomass acclimatization could be very important to achieve higher H<sub>2</sub> production and higher percentages of substrate consumption (Abreu *et al.*, 2009).

#### 2.8.4 Inocula source

Inocula from various sources such as municipal and industrial anaerobic bioreactors, compost, cow dung, agriculture soil, and isolated bacterium have been investigated for H<sub>2</sub> production. Mixed cultures from anaerobic and even aerobic environments (activated sludge) were examined (Xiea *et al.*, 2008) and the effects of substrate, environmental, and operational conditions were shown to be affected by the culture source. The type of the reactor can affect the physical structure of the sludge (flocculent, suspended, or granular).

Mixed cultures from different sources contain different proportions of the various trophic groups depending on the type of substrate and the environmental conditions in their original sources (Moreno-Andrade and Bultron, 2004). Activities of substrate degradation varied widely in cultures from different sources under comparable environmental conditions (Table 2.8). For these reasons, various types of cultures from different sources have been investigated in H<sub>2</sub> production studies (Table 2.4) mainly to examine the effects of enrichment pretreatment methods.

The effects of heat-treatment and BES on granular sldge form an upflow anaerobic sludge blanket reactor (UASB) treating brewery wastewater and suspended cultures from an anaerobic digester in a municipal wastewater treatment plant have been examined by Danko *et al.* (2008) and Abreu *et al.* (2009). These researchers attributed the differences in H<sub>2</sub> production to differences in the microbial communities from different origins. Kawagoshi *et al.* (2005) examined the effect of heat and acid pretreatments on H<sub>2</sub> yield in activated sludge, digested sludge, refuse compost, lake sediments, and agricultural soils from kiwi and watermelon fields. They found that the effect was different depending on the inocula types.

Although LCFAs' inhibition on methane fermentation in cultures from different origins has been reported by Hwu *et al.* (1996) their effects on H<sub>2</sub> production in cultures from different origins have not been reported.

## **2.9 MOLECULAR BIOLOGY TECHNIQUES**

The microbial communities are a dynamic factor affecting the carbon flow in metabolic networks. The best method for determining the composition of H<sub>2</sub> producing mixed cultures under heterogeneous conditions is still not resolved and previous traditional microbiological culturing methods such as Most Probable Number (MPN) and plate count have been used to quantify species in anaerobic consortia (Toerien *et al.*, 1967). These methods are limited because less than 1% of the microorganisms could be grown on selective laboratory media (Amann *et al.*, 1995). Only about 8200 valid cultivated species are known (see <http://www.bacterio.cict.fr> for updates) (Euzéby, 1997). Molecular biology methods using 16S rRNA gene sequence have also been used to study the dynamics of microbial mixed cultures. The 16S rRNA sequence database includes approximately 500,000 sequences of more than 300 bp (see [www.arb-silva.de](http://www.arb-silva.de)). This has led to an increase of the probability of 16S rRNA gene-based identification of unknown species (Von Wintzingerode *et al.*, 2002; Cole *et al.*, 2007; Marsh, 1999). Molecular biology methods such as T-RFLP, DGGE, and FISH are all fingerprinting techniques which produce microbial community profiles, but they differ in their resolution.

### **2.9.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)**

The use of fingerprinting to generate a microbial community profile is possible by analyzing the polymorphism of a specific gene using the T-RFLP technique. T-RFLP is a robust, sensitive, and reproducible semi-quantitative community profiling method

based on the 16S rRNA gene (Clement *et al.*, 1998). It can be used to compare samples and assess similarities. It is currently one of the most rapid and powerful methods in microbial ecology to compare spatial and temporal changes in the bacterial community structure (Wang *et al.*, 2004). Thus, it has been used to characterize anaerobic microbial communities phylogenetically (Kotsyurbenko *et al.*, 2007; Rossetti *et al.*, 2003). T-RFLP has better resolution than DGGE or TGGE. The terminal restricted fragments (T-RFs) obtained are differentiated by size on high resolution ( $\pm 1$  bp) sequencing gels (Tiedje *et al.*, 1999). The result of T-RFLP is a digital profile which includes the peak (fragment) height, area, and size in graphical and tabulated forms. The output is simple digital information about the species and the intensity of fluorescence (relative abundance) of the various community members. Thus, comparisons of different communities become easier (Park *et al.*, 2006).

Figure 2.5 shows the steps involved in the T-RFLP method. The extracted DNA is PCR amplified and labeled with a fluorescently end-labeled primer set (Marsh, 1999).

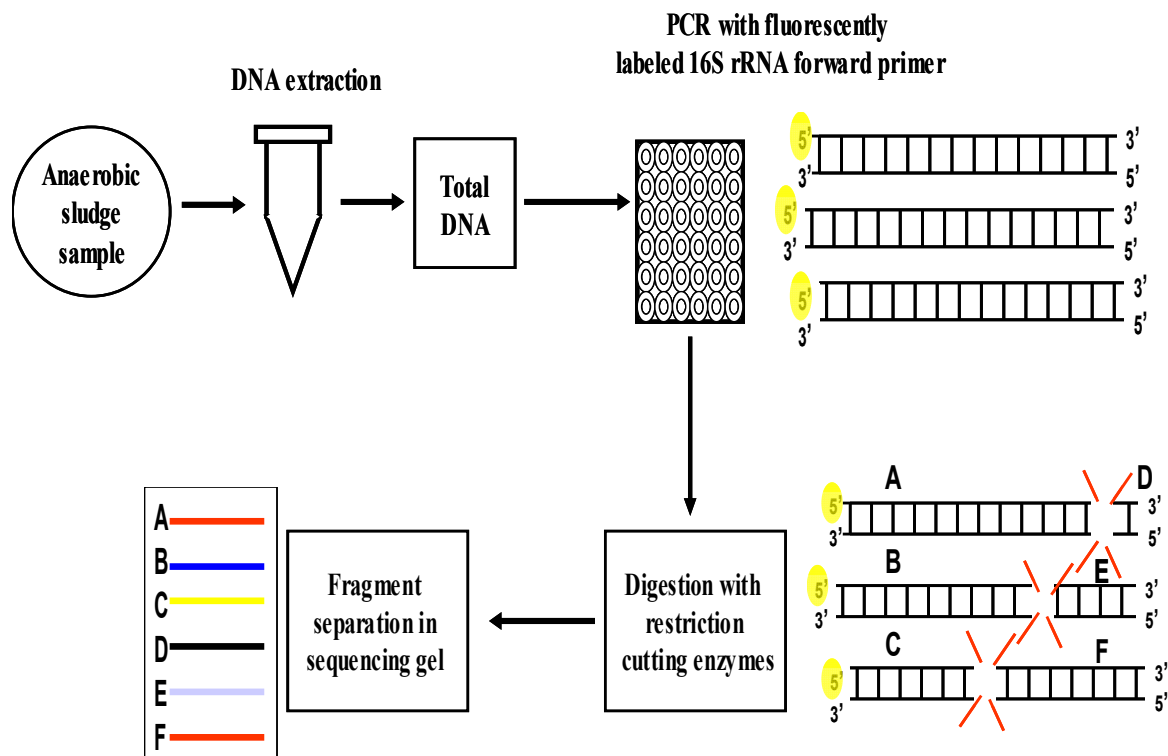


Figure 2.5 Main steps of T-RFLP analysis.



The labeled DNA is then digested with restriction enzymes. The use of several restriction enzymes increases confidence in the phylogenetic identification (Tiedje *et al.*, 1999). The terminal restriction site for each species in the community is different because of variation in the sequences. By limiting analysis to a fluorescently-labeled terminal restriction fragment (T-RF), each species gives one T-RF which can be used to detect the existence and quantify the abundance of particular DNA sequences in a mixture of amplicons.

Resolution of T-RFLP can be optimized to identify individual species in a diverse community. The results of T-RFLP depend on the selection of the restriction enzyme. Different restriction enzymes cut the same sequence at different locations. The results differ significantly from enzyme to enzyme. Therefore, the selection of restriction enzymes should be optimized for the purpose of study. T-RFLP is cost effective in comparison to other molecular biology methods (Kim *et al.*, 2010).

## **2.10 FEEDSTOCK**

Mixed culture dark hydrogen-fermentation could accommodate a vast range of potential organic substrates. In industrial application, the criteria for selecting a substrate are availability, cost, and biodegradability (Kapdan and Kargi, 2006). Various synthetic and actual organic wastes have been used in H<sub>2</sub> fermentation. However, carbohydrate-rich substrates produced 20 times more H<sub>2</sub> than fat- and protein-rich substrates (Lay *et al.*, 2003).

In sections 2.3.1 and 2.3.3, H<sub>2</sub> yields from pure carbohydrates such as glucose, xylose, arabinose, sucrose, starch, lactose and cellulose have been reported (Table 2.3 and 2.4). Glucose and sucrose were the most widely investigated substrates followed by starch and cellulose (Li and Fang, 2007). The maximum yields reported can vary even for the same substrate depending on environmental and operation conditions,

type of bioreactor, and use of pretreatments or inhibitors. Effluents from industries such as, for example, sugar factories, rice wineries, palm oil mills, chemical plants, food-processing plants, and domestic sewage (Shin, *et al.*, 2004) have been used as substrates for H<sub>2</sub> production (Table 2.4). The H<sub>2</sub> yields from sugar factories, palm oil mills, and food processing facilities were 2.52, 2.03, and 1.95 mol H<sub>2</sub>·mol<sup>-1</sup> hexose, respectively. Therefore, they are comparable to yields obtained from glucose (Table 2.4). These results are important because they indicate dark fermentation could produce clean biofuels using feedstock chemicals from a variety of industrial effluents. Hydrogen production from solid wastes such as kitchen, food processing, mixed wastes, and municipal wastes has been reported in a large number of studies (Li and Fang, 2007). According to Valdez-Vazquez *et al.* (2005), the highest H<sub>2</sub> yield was 437 ml H<sub>2</sub> per gram hexose (3.5 mol H<sub>2</sub>·mol<sup>-1</sup> glucose) when solid waste was used as a feedstock

## **2.11 BIOREACTORS FOR HYDROGEN PRODUCTION**

Generally, H<sub>2</sub> can be produced in batch and continuous fermentation; however, industrial application requires continuous or at least semi-continuous processes (Hallenbeck and Gosh, 2009). An optimized continuous reactor would provide robust, reliable, stable performance over long-term operation, reduce temporary fluctuations in operational parameters, and increase volumetric production rates (Hallenbeck and Gosh, 2009). Many types of continuous reactors (Table 2.9) have been investigated to improve H<sub>2</sub> production. The CSTR is the most commonly used reactor because it allows for effective pH and temperature control (Li and Fang, 2007). However, in CSTR the hydraulic retention time (HRT) controls the microbial growth rate and consequently, H<sub>2</sub> production is limited (Li and Fang, 2007). Reactors employing physical retention (immobilization) of microbial biomass uncouple the

HRT and cell growth rate. Immobilized or attached growth systems increases the volumetric H<sub>2</sub> production rate up to >50-fold over that of CSTR and allowed high volumetric loading rates because of the high cell concentrations which can be retained (Hallenbeck and Gosh, 2009). However, the H<sub>2</sub> yields from these reactors are not greater than those from CSTR. It is unclear whether the differences in the results from different reactors (Table 2.9) are due to different configurations or differences in operational parameters. Overall, H<sub>2</sub> consumption related to methane production is a potential problem for these reactors because cell growth is not controlled by HRT; hence, methanogens can grow even at high rates of liquid flow (Hallenbeck and Gosh, 2009).

Table 2.9 Reactor configurations in H<sub>2</sub> dark fermentation.

Reactor type	Substrate	HRT (h)	H <sub>2</sub> yield <sup>a</sup>	H <sub>2</sub> production rate <sup>b</sup>	Reference
Continuously stirred tank bioreactor (CSTBR)	Carbohydrates	4	1.18 <sup>c</sup>	0.91	Wu <i>et al.</i> , 2007
Anaerobic sequencing batch reactor (ASBR)	Glucose	8	0.37	0.23	Cheong <i>et al.</i> , 2007
Fixed bed bioreactor with activated carbon (FBBAC)	Sucrose	1.0	NR	1.32	Chang <i>et al.</i> , 2002
Upflow anaerobic sludge blanket reactor (UASB)	Sucrose	8	1.5	1.96	Chang and Lin., 2004
Polymethymethacrylate (PMMA) immobilized cells	Sucrose	6	2.0	1.8	Wu and Chang, 2007
Carrier-induced granular sludge bed reactor (CIGSB)	Sucrose	4	4.02	9.3	Lee <i>et al.</i> , 2006
Fluidized bed reactor (FBR)	Sucrose	4	1.04	1.32	Wu <i>et al.</i> , 2007
Anaerobic fluidized bed reactor (AFBR)					
- Biofilm	Glucose	0.25	0.4	7.6	Zhang <i>et al.</i> , 2008
- Granules	Glucose	0.25	1.7	6.6	
Continuously stirred anaerobic bioreactor (CSABR)	Sucrose	0.5	3.5	15	Wu <i>et al.</i> , 2007
Trickling biofilter reactor (TBR)	Glucose	12	1.11	0.98	Oh <i>et al.</i> , 2004

Notes: **a**- the unit = mol·mol<sup>-1</sup> substrate; **b**- the unit = L H<sub>2</sub> L<sup>-1</sup> h<sup>-1</sup>; **c**- the unit = mol·mol<sup>-1</sup> hexose.

## **2.12 OPERATIONAL CONDITIONS AFFECTING HYDROGEN PRODUCTION**

### **2.12.1 Temperature**

Many studies investigated the effects of temperature on fermentative H<sub>2</sub> production (Tables 2.3 and 2.4). Temperature affects the growth rate of bacteria and the rate of enzymatic reactions, and thus influences H<sub>2</sub> production and yield. Optimal temperatures for H<sub>2</sub> production in mesophilic (30-37°C) and thermophilic (40-85°C) conditions as well as ambient room temperature have been investigated in numerous studies.

Acidogenic cultures can produce more H<sub>2</sub>, less propionate and no methane under thermophilic in comparison to mesophilic conditions (Shin *et al.*, 2004). Mu *et al.* (2006) reported that the rate of H<sub>2</sub> production increased with increasing temperature from 33 to 39 °C, then decreased as the temperature increased to 41 °C. The temperature variation also influenced the distribution of aqueous products (Mu *et al.*, 2006). Lin and Chang, (2004) concluded that microbial communities in sewage sludge could produce H<sub>2</sub> within six intervals (4 °C) of uncontrolled ambient temperature (15 to 34 °C range). However, under controlled temperature conditions, the yield increased and then attained a stabilized value.

### **2.12.2 Hydraulic Retention Time (HRT)**

HRT is an important design parameter, which determines the economics of operating a reactor. A short HRT leads to a smaller and more economical bioreactor for a given flow rate. During continuous H<sub>2</sub> production in a continuous fed CSTR without recycle, the solids retention time (SRT) is the same as the HRT. In this type of reactor configuration, the design HRT will influence the microbial population distribution and hence, the metabolic by-products. For example, if the growth rate of

a species is less than the HRT, then its population will be washed out from the reactor. Iyer *et al.* (2004) reported that the microbial diversity increases with HRT values. They reported that slow growing *Bacillaceae* and *Enterobacteriaceae* were washed out at HRT of 10 h and only *Closteridiaceae* were present. Under these conditions, the  $H_2$  yield reached a value of  $1.61 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ glucose}$  (production rate of  $436 \text{ ml } H_2 \text{ h}^{-1}$ ).

In systems with a cell recycle, long SRT ( $>12\sim24 \text{ h}$ ) are achievable and slow growing  $H_2$  consuming methanogens are not washed out from the reactor (Chen *et al.*, 2001). Moreover, *Clostridia* can shift metabolite production to alcohols at low pH values (less than 5) and low growth rates (Dabrock *et al.*, 1992). The  $H_2$  production and  $H_2$  percentage increased when HRT decreased at long HRT and decreased with a HRT decrease at short HRT (2 h) (Chen and Lin, 2003). In general, the optimal HRT for simple substrates such as glucose and sucrose were in the range of 3-8 h, however, HRT as short as 1 h (Chang *et al.*, 2002) and as long as 13.7 h (Fang and Liu, 2004) have been reported. Chen and Lin (2003) reported  $H_2$  yield of  $4.52 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ sucrose}$  in CSTR with HRT of 8 h. For complex substrates such as starch and food waste, long HRT ( $>12 \text{ h}$ ) are needed to account for the slow initial step of hydrolysis (Lay, 2000). At pH 5.2, the optimal HRT reported for starch was 15 h with  $N_2$  sparging at  $30^\circ\text{C}$  (Hussy *et al.*, 2003) and 17 h and  $37^\circ\text{C}$  (Lay, 2000). For effluents from a full-scale rice winery facility, the optimal HRT was 2 h (Yu *et al.*, 2002) and 12 h for sugar factory wastewater (Ueno *et al.*, 1996). Solid food wastes required longer HRT (120 h) (Shin and Youn, 2005).

HRT can be uncoupled from SRT in reactors using immobilized biomass such as upflow anaerobic sludge blanket (UASB) and anaerobic filter (AF). Long SRT can be maintained while applying short HRTs. Stable  $H_2$  production at HRT ( $< 2 \text{ h}$ ) was reported by Lee *et al.* (2004) in a carrier-induced granular sludge bed (CICGB)

bioreactor; however, the yield was low ( $1.23 \text{ mol H}_2 \cdot \text{mol}^{-1}$  sucrose). Nevertheless, although short HRT inhibits methanogenesis because  $\text{H}_2$  producing bacteria grow faster than methanogens, it cannot prevent  $\text{H}_2$  loss due to homoacetogenesis.

### **2.12.3 Effects of volatile fatty acids**

Acetate and butyrate are the major acids produced in balanced anaerobic digestion, whereas under stress conditions propionate and lactate are produced. These VFAs exert both product and substrate inhibition on acetogens and methanogens, respectively. Acetate,  $\text{H}_2$  or propionate can inhibit the fermentation of propionate to methane (Fukuzaki *et al.*, 1990a, 1990b). High concentrations of acetate inhibit aceticlastic methanogenesis (Fukuzaki *et al.*, 1990b; Lokshina *et al.*, 2001).

A bacterial cell maintains its internal pH near neutrality. Free (undissociated) non-polar acids can penetrate the cell membrane at low pH. At the higher pH inside the cell, they dissociate, release protons, and uncouple the proton motive force (Horn *et al.*, 2003). This increases the energy required to maintain the intracellular pH near neutrality (Jones and Woods, 1986). In addition, it decreases coenzyme A and phosphate pools which in turn decrease the flux of glucose through glycolysis (Gottwald and Gottschalk, 1985). For example, acetic acid exists in its free form at  $\text{pH} < 4.7$ . Undissociated acids cause the fermentation to switch from  $\text{H}_2$  to solvent production (Gottwald and Gottschalk, 1985; Jones and Woods, 1986). A total concentration of 19 mM of self-produced undissociated acids decreased the  $\text{H}_2$  yield significantly and initiated solventogenesis (Van Ginkel and Logan, 2005). Self-produced acids are more inhibitory than externally-added acids of the same concentrations (Van Ginkel and Logan, 2005).

#### **2.12.4 Hydrogen partial pressure**

Hydrogen partial pressure ( $P_{H_2}$ ) affects  $H_2$  production (Mizuno *et al.*, 2000a). The increase of  $H_2$  concentration in the liquid phase renders the reactions by which bacteria re-oxidize the reduced ferredoxin and  $H_2$  carrying coenzymes thermodynamically unfavorable (Ruzicka, 1996). Therefore, lowering the  $P_{H_2}$  should enhance  $H_2$  production. Tanisho *et al.* (1998) and Mizuno *et al.* (2000a) reported that lowering dissolved  $H_2$  by sparging with  $N_2$  caused a 68% increase in the  $H_2$  yield. Kim *et al.* (2004) also reported that  $H_2$  production was enhanced by sparging with  $CO_2$ . Pauss and Guiot (1993) observed that the  $H_2$  mass transfer from liquid to gas phases is extremely limited and the dissolved  $H_2$  value is far from the equilibrium value. Therefore, application of a stirring regime and increasing agitation speed (from 100 to 700 rev/min) doubled the daily rate of  $H_2$  production (Lay, 2000). According to Lay (2000), the dissolved  $H_2$  concentrations may be related to substrate concentration.

#### **2.12.5 Nutrients and inhibitors**

Nutrients and inhibitors influence dark fermentative  $H_2$  production as they do in any biological process. Microorganisms require substrates, nutrients, and metals in quantities which promotes optimum growth. Excessive concentrations of substrates, nutrients, or metals can impose inhibitory action on growth. Ammonia (nitrogen), cations, heavy metals, and volatile fatty acids are the most important substances under this category (Pavlostathis and Giraldo-Gomez, 1991). This section summarizes the reported finding regarding the concentrations of these substances in  $H_2$  fermentation studies.

Ammonia inhibits  $H_2$  production, but reports of the optimal level of nitrogen 0.1 to 2.0 g  $L^{-1}$  N) or carbon/nitrogen (C/N) ratio (3.3 to 130) vary widely (Liu and Fang,

2007). Wu *et al.* (2007 and 2008) reported  $5.24 \text{ g L}^{-1} \text{ NH}_4\text{HCO}_3$  and  $3.55 \text{ g L}^{-1} \text{ NH}_4\text{Cl}$  in the growth media. A period of acclimation can attenuate the inhibitory effect of nitrogen. Reactors acclimated to low concentrations of ammonia ( $<0.8 \text{ g L}^{-1} \text{ N}$ ) produced  $\text{H}_2$  continuously at high N concentrations ( $<7.8 \text{ g L}^{-1}$ ) (Salerno *et al.*, 2006).

Phosphate is used as both a nutrient and buffer agent (Oh *et al.*, 2003; Lin and Lay, 2005). Concentrations of  $\text{K}_2\text{HPO}_4$  as high as 40 and  $20 \text{ g L}^{-1}$  were reported by Fan and Chen (2004) and Tanisho *et al.* (1998), respectively. An optimal ratio of carbon/phosphate of 120-130 was reported (Hawkes *et al.*, 2002; Lin and Lay, 2005).

Some metal ions affect the activity of  $\text{H}_2$ -producing bacteria because they are essential, but can have an inhibitory effect if the optimal levels are not used. Magnesium (Mg), sodium (Na), zinc (Zn), and iron (Fe) are important for  $\text{H}_2$  production in a descending order (Lin and Lay, 2005).  $\text{Mg}^{2+}$  activates 10 enzymes in glycolysis; inadequate Mg limits growth (anabolism) and the  $\text{H}_2$ -producing ability of fermentative bacteria (Liu *et al.*, 2008). Iron is essential for hydrogenase (Junelles *et al.*, 1988; Hawkes *et al.*, 2002); a shortage of iron affects growth, metabolism, lowers the production of  $\text{H}_2$  and VFAs, and increases alcohol production (Junelles *et al.*, 1988; Lee *et al.*, 2001).  $\text{Fe}^{2+}$  is known to increase the activity of specific enzymes (e.g, NADH-Fd reductase) in  $\text{H}_2$  producing fermentative bacteria, and to enhance their  $\text{H}_2$  producing ability (Liu *et al.*, 2008). Adding  $\text{Fe}^{2+}$  could shift the fermentation from butyric acid to ethanol (Wang *et al.*, 2003), however; the reported optimal  $\text{Fe}^{2+}$  concentration for  $\text{H}_2$  production varies from  $10 \text{ mg L}^{-1} \text{ Fe}^{2+}$  (Liu and Shen, 2004) to  $353 \text{ mg L}^{-1} \text{ Fe}^{2+}$  (Lee *et al.*, 2001).

The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) of zinc ( $4.5 \text{ mg L}^{-1}$ ) was less than copper ( $6.5 \text{ mg L}^{-1}$ ) and chromium ( $60 \text{ mg L}^{-1}$ ) (Li and Fang, 2007). Yu and Fang (2001) found Copper more toxic than zinc. According to Zheng and Yu (2004), 500



mg L<sup>-1</sup> and 400 mg L<sup>-1</sup> of zinc and copper, respectively, did not affect the H<sub>2</sub> yield adversely, but increased the lag phase.

## **2.13 PRINCIPAL COMPONENTS ANALYSIS (PCA)**

Principal Components Analysis (PCA) is a multivariate statistical method used in this research to reduce data, visualize quantitative relationships among complex multidimensional data sets, detect similarities and differences between different data sets, enable better interpretation of the data, and extraction of conclusions (Wise and Gallagher, 1996). Most importantly, PCA can provide information on the most meaningful parameter in the data set without any loss or change in the original data (Helena *et al.*, 2000).

Technically, PCA replaces the original multidimensional variables ( $n$ ) in the data set by a number of new artificial uncorrelated linear combinations of variables ( $m$ ) called principal components (PCs) ( $m < n$ ) (Lee *et al.*, 2006); therefore, PCA eliminates any redundancy (correlation between variables). Each PC is a linear combination of optimally-weighted observed variables. The first PC accounts for the maximum total variance in the data set, the second PC accounts for the next largest variation not already accounted for by the first PC, and so on. Generally, as the correlation among the original variables increases, the number of PCs generated decreases. Usually the first two PCs account for most of the variation in the data set. Consequently, the data set can be represented in only two instead of the original ( $n$ ) dimensions. The graphical representation of the plane PC1-PC2 includes the most information of all planes that could be drawn through the data in the  $n$ -dimensional space.

Results of the PCA are given as score and loading maps. The score maps display observations to show groups, trends, outliers and similarities among samples.

Variables that have higher coefficients than others (positive or negative) on certain PC are important for the score given to that PC. The loading maps show which variables are most important for the differences observed among samples. The loading plots detect correlations between variables (Massart and Vander Heyden, 2005).

The score plots of PCA revealed differences between microbial communities in previous studies (Wikstroëm *et al.*, 1999). PCA evaluation of DNA fingerprints (FPs) derived from bioreactor cultures produced unbiased, easily visualized profiles of the development of complex microbial communities (Wikstroëm *et al.*, 1999).

PCA has been applied to data sets from H<sub>2</sub> production studies (Abreu *et al.*, 2009) and microbiological T-RFLP's profiles (Park *et al.*, 2006). Combining T-RFLP with PCA was shown to be an effective strategy for comparing different microbial communities and detecting the major changes (Wang *et al.*, 2004).

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## CHAPTER 3: MATERIALS AND METHODS

### 3.1 INOCULUM SOURCE

The inoculum cultures were procured from industrial and municipal wastewater treatment reactors. Granulated cultures were obtained from an upflow anaerobic sludge blanket reactors (UASBRs) from a brewery (Guelph, ON) and an ethanol production plant in (Chatham, Ontario). Flocculated cultures were obtained from anaerobic digesters located in municipal wastewater treatment facilities (Chatham, ON).

All the cultures were maintained in 4-L semi-batch reactors with temperature maintained at  $37\pm 2^{\circ}\text{C}$  using thermal tape (TP FG STD, Omegalux, USA) wrapped around the reactor's wall and a variable transformer for temperature control (Staco, Inc. Ohio). All cultures were fed  $5\text{ g L}^{-1}$  glucose every 6 to 7 days. The cultures were characterized for VSS/TSS (APHA *et al.*, 1998), glucose removal, VFAs and gas production. All characterization studies were conducted in 160 mL serum batch reactors ( $2,000\text{ mg L}^{-1}$  VSS and fed  $5,000\text{ mg L}^{-1}$  glucose (Spectrum Chemicals, CA) at initial pH 7.6 prepared according to the protocol described in section 3.2. The cultures were used in the experiment only after the results of the characterization study confirm that the cultures were able to remove  $5000\text{ mg L}^{-1}$  glucose within 24 hours and the liquid and gas by-products were completely converted into  $\text{CH}_4$  and carbon dioxide within 6 to 7 days. A sample of the characterization data are given in Appendix A.

### 3.2 SERUM BOTTLES PREPARATION PROTOCOL (BATCH REACTORS)

All the experimental methods reported in this work were adapted from past studies (Lalman and Bagley, 2000; 2002; Lalman *et al.*, 2004). Serum bottles (160 mL) were filled with 50 mL of basal media plus culture (2,000 mg L<sup>-1</sup> VSS) in an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI) under 80% N<sub>2</sub>/20% CO<sub>2</sub> (Praxair Inc. ON). The composition of the basal medium (pH: 8.0-8.2; adapted from Weigant and Lettinga, 1985) is given in Table 3.1. All chemicals were lab grade (Spectrum Chemicals, CA). Plate 3.1 shows the steps of the experimental work and the main instruments.

Table 3.1 Composition of the basal media.

Ingredient	Concentration (mg L <sup>-1</sup> )
NaHCO <sub>3</sub>	6000
NH <sub>4</sub> HCO <sub>3</sub>	70
KCl	25
K <sub>2</sub> HPO <sub>4</sub>	14
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	10
Yeast extract	10
MgCl <sub>2</sub> .4H <sub>2</sub> O	9
FeCl <sub>2</sub> .4H <sub>2</sub> O	2
Resazurin	1
EDTA (ethylenediaminetetraacetic acid)	1
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.5
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.15
NaSeO <sub>3</sub>	0.1
(NH <sub>4</sub> ) <sub>6</sub> MoO <sub>7</sub> .4H <sub>2</sub> O	0.09
ZnCl <sub>2</sub>	0.05
H <sub>3</sub> BO <sub>3</sub>	0.05
NiCl <sub>2</sub> .6H <sub>2</sub> O	0.05
CuCl <sub>2</sub> .2H <sub>2</sub> O	0.03

After allowing the solids to settle for 5 minutes, a known volume of liquid equivalent to the amount of substrate to be added was removed from each bottle. Varying volumes of each substrate (glucose and LA) were added to maintain a total liquor volume of 50 mL in each bottle depending on the experimental condition under

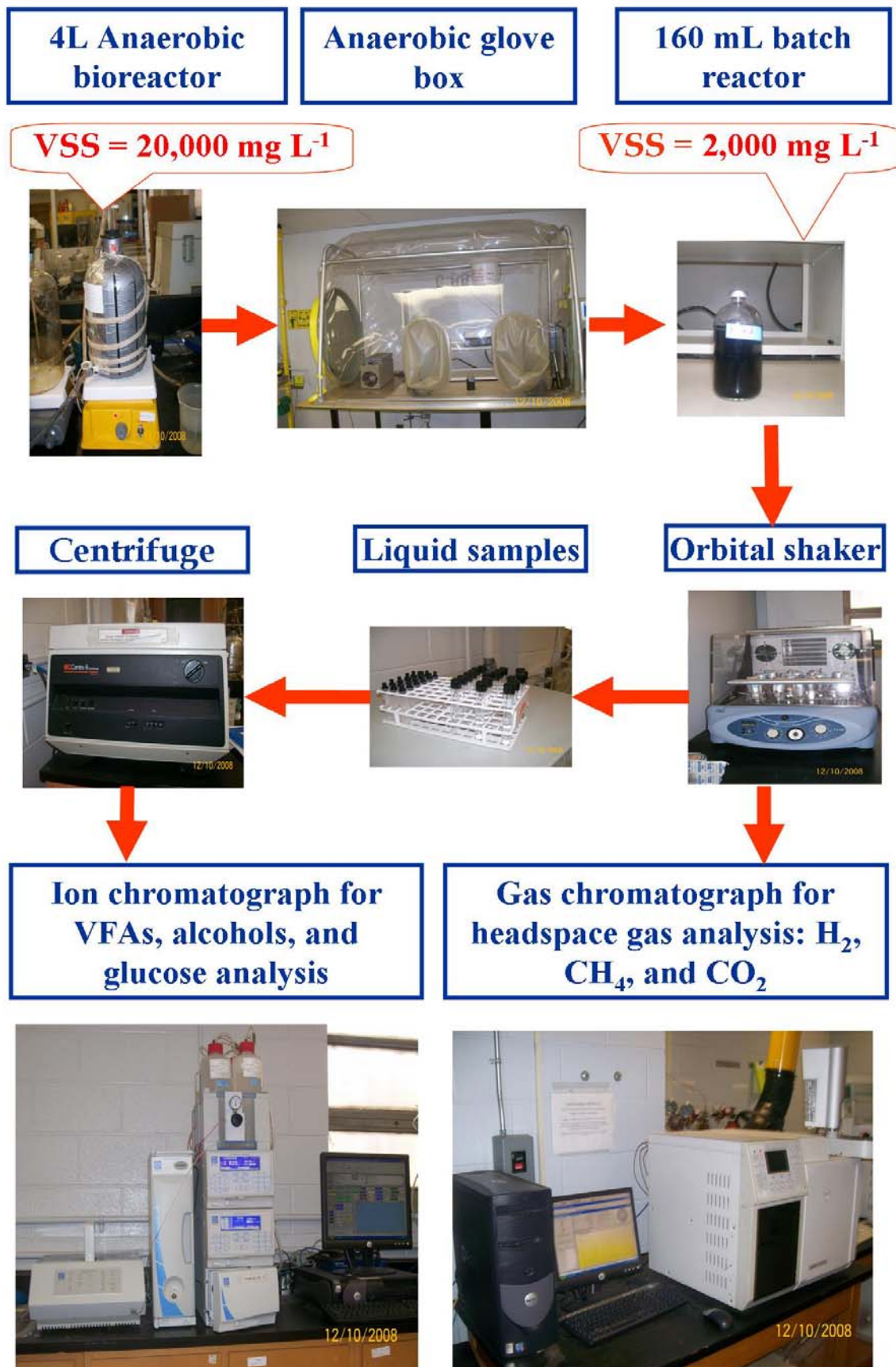


Plate 3.1 Schematic of the experimental methods.

study. The initial pH was adjusted inside the glove box according to the experimental design using 1 M HCl or 1 M NaOH. The bottles were placed in an anaerobic glove box and sealed with Teflon<sup>®</sup>-lined silicone rubber septa and aluminum crimp caps. Each bottle was over-pressurized with 20 mL of 80% N<sub>2</sub>/20% CO<sub>2</sub> prior to the addition of any substrate (glucose or LCFA) to prevent the formation of a negative pressure in the headspace during sampling. Any residual hydrogen (H<sub>2</sub>) injected into the headspace from the glove box atmosphere was removed by agitation at 200 rpm for 24 hours at 37°C (Lab Line Instruments Model 3520, IA) before adding the carbon source (glucose) to the serum bottles.

All the LCFAs stock solutions (100,000 mg L<sup>-1</sup>) for injection into the culture bottles was prepared using LCFA melted *au bain-marie* in hot NaOH (Rinzema *et al.*, 1994). LCFAs are insoluble (Table 3.2; Ralston and Hoerr, 1942) and an immediate inhibitory response on the microbial population is unlikely. Hence, LCFAs were added to the culture 24 hours before adding glucose.

Table 3.2 LCFAs used in the experiments.

<b>LCFA</b>	<b>Carbon number</b>	<b>Abbreviation</b>	<b>Solubility in water* (mg L<sup>-1</sup>)</b>	<b>Formula</b>
<b>Lauric</b>	12	C12:0	0.55 to 0.63	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>10</sub> COOH
<b>Myristic</b>	14	C14:0	0.24 to 0.29	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>12</sub> COOH
<b>Palmatic</b>	16	C16:0	0.083 to 0.10	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>14</sub> COOH
<b>Linoleic</b>	18	C18:2	0.034 to 0.042	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>16</sub> COOH

\* Solubility is at 30 and 45°C (Ralston and Hoerr, 1942)

### 3.3 KINETIC CHARACTERIZATION PROCEDURE

Maximum specific substrate utilization activity and the percentage of the corresponding active biomass were determined using serum bottle batch reactors. The serum bottle batch reactors were fed repeatedly with one substrate and a degradation profile was developed by periodic monitoring after repeated successive injections.

The linear degradation curve of each substrate was used to estimate proportion of the active biomass and the specific substrate utilization rate. Each condition was examined in triplicate. The substrates used were glucose, lactate, acetate, butyrate, propionate, and hydrogen. All kinetic characterizations were conducted at  $37 \pm 1^\circ\text{C}$  with an initial pH of 7.6 in phase II, whereas the initial pH in phase III was 5.

### **3.4 EXPERIMENTAL DESIGN**

#### **3.4.1 Phase I - Effects of LA on H<sub>2</sub> production using microbial cultures from different sources**

Experiments were designed to examine the effects of the culture source on H<sub>2</sub> production, and to assess the effects of LA on H<sub>2</sub> production from glucose using cultures from different sources. It was not clear what the response of cultures from different sources would be to LA, and to what extent they would differ or resemble each other in terms of H<sub>2</sub> production in the presence of LA.

The following three cultures from different sources were used: 1) granular sludge from a UASB reactor at a brewery (Chatham, ON); 2) granular sludge from a UASB in an ethanol producing plant (Chatham, ON); and 3) flocculated a secondary digester in a municipal wastewater treatment plant (Chatham, ON). The inocula were maintained in 4 L semi-continuous reactors at  $37^\circ\text{C}$  and were fed glucose ( $5 \text{ g L}^{-1}$ ) every 6-7 days.

All experiments were conducted in 160 mL serum bottle batch reactors incubated at ( $37 \pm 2^\circ\text{C}$ ) with an initial pH of 5, and VSS of  $2,000 \text{ mg L}^{-1}$ . Each culture sample was incubated for a total period of 10 days, with 2 successive glucose injections (on day 0 and 5, respectively). For each culture, the following experiments were conducted (Table 3.3):

- 1- Glucose control, 2- LA control, and 3- Incubation with glucose plus LA.

Triplicates glucose control batch reactors were prepared for Experiment 1. For Experiments 2 and 3 (i.e. LA control and incubation with glucose plus LA), two sets of triplicate batch reactors were prepared and injected with LA (2,000 mg L<sup>-1</sup>) (TCI, USA) one day before day 0. Glucose (5,000 mg L<sup>-1</sup>) (ACP Chemicals, Montreal, Quebec) was injected on day 0 and again on day 5 in the glucose experiments (i.e. Experiment 1 - glucose control and Experiment 3 - glucose plus LA). Five days after the first glucose injection (i.e. Day 5), all batch reactors were opened, the pH was re-adjusted to the initial value (pH 5), purged with nitrogen (99.998 %) for 3 minutes and re-sealed again with Teflon lined septa capped with aluminum crimp.

Headspace gases, VFAs, and alcohols were monitored daily for 5 days after each glucose injection for all batch reactors. Glucose degradation was monitored for 24 hrs in the glucose control and glucose plus LA experiments. Five days after the second glucose injection (i.e. Day 10), all serum bottle batch reactors were opened and VSS/TSS measurements were conducted.

Table 3.3 Experimental design matrix: effect of inocula source on hydrogen production.

sludge	Substrate fed		Liquid and Gas Analysis			
	LCFA (mM)	Glucose (5 g L <sup>-1</sup> )	Glucose	VFAs	Alcohols	H <sub>2</sub> , CH <sub>4</sub> and CO <sub>2</sub>
<b>Brewery sludge (A)</b>	X	√	√	√	√	√
	√	√	√	√	√	√
	√	X	X	√	√	√
<b>Ethanol sludge (B)</b>	X	√	√	√	√	√
	√	√	√	√	√	√
	√	X	X	√	√	√
<b>Municipal sludge (C)</b>	X	√	√	√	√	√
	√	√	√	√	√	√
	√	X	X	√	√	√



### 3.4.2 Phase II - Effects of adaptation to glucose on culture structure, composition, and activity

Experiments for Phase II were designed in accordance to Table 3.4. In this study, the three cultures were characterized to determine their maximum specific activities for glucose, lactate, acetate, butyrate, propionate, and H<sub>2</sub>, and to estimate the proportion of the corresponding active trophic groups responsible for the measured activities. Also, the effects of adaptation to glucose on the specific activities and the composition of the active biomass were assessed by comparing the kinetics of the cultures after one year of adaptation. Six sets of triplicate batch reactors were prepared for each of the cultures (A, B, and C) according to the protocol described in section 3.2.

Table 3.4 Experimental design matrix: kinetic characterization of cultures A, B, and C.

Substrate	Type of sludge					
	Brewery sludge (A)		Ethanol sludge (B)		Municipal sludge (C)	
	Adapted	Non-adapted	Adapted	Non-adapted	Adapted	Non-adapted
Glucose	√	√	√	√	√	√
Lactate	√	√	√	√	√	√
Acetate	√	√	√	√	√	√
Propionate	√	√	√	√	√	√
Butyrate	√	√	√	√	√	√
H <sub>2</sub>	√	√	√	√	√	√

### 3.4.3 Phase III - Effects of linoleic acid and pH on the population and activity of the various trophic groups and H<sub>2</sub> yield in mixed culture.

In phase III, experiments were designed (Table 3.5) to characterize the mixed cultures under increasing stress conditions, to determine their maximum specific activities for glucose, lactate, acetate, butyrate, propionate, and H<sub>2</sub>, and to estimate the percentage of the corresponding active trophic groups responsible for the measured activities.

Table 3.5 Experimental design matrix for phase III.

Substrate	Stage I (Control pH 7.6)		Stage II (pH 5)		Stage III (pH 5)		Stage IV (pH 5+LA)	
	Started a week before stage II		Started on day 0		Started on day 0 (H <sub>2</sub> production study for 8 days)		On day 0 all bottles of stage IV were injected with LA, and have been left incubated for 8 days until monitoring Stage II bottles finished, then Stage IV bottles were injected the substrate indicated and the kinetic study in the presence of LA was started.	
	Substrate	LA	Substrate	LA	Substrate	LA	Substrate	LA
Glucose	√	0	√	0	√	√	√	√
					√	0		
Lactate	√	0	√	0			√	√
Acetate	√	0	√	0			√	√
Propionate	√	0	√	0			√	√
Butyrate	√	0	√	0			√	√
H <sub>2</sub>	√	0	√	0			√	√

The mixed culture used in this study was culture B (from ethanol production facility) and it was maintained according to the protocol described in section 3.1. This phase included four stages of experimentation (each stage had its own sets of batch reactors, but the type/source of sludge has been used in all stages). At the end of each stage the bottles were sacrificed to determine the VSS concentration. The four stages were as follows:

- Stage I: Kinetic characterization of six triplicate sets of the mixed cultures at pH 7.6 for one week (Control condition).
- Stage II: Kinetic characterization of six triplicate sets of the mixed cultures at pH 5.
- Stage III: Kinetic characterization of six triplicate sets of the mixed cultures at pH 5 plus 2,000 mg L<sup>-1</sup> LA.
- Stage IV: Hydrogen production from glucose in the presence of 2,000 mg L<sup>-1</sup> LA and initial pH 5.

In Stages I, II and IV, the kinetic characterized has been conducted according to the protocol described in section 3.3. All serum batch reactors were prepared according to the protocol described in section 3.2. All experiments were conducted in 160 mL serum bottle batch reactors incubated at  $(37\pm 2^{\circ}\text{C})$  with an initial pH of 5 (except stage I where  $\text{pH} = 7.6$ ), and VSS  $2,000 \text{ mg L}^{-1}$ .

In the  $\text{H}_2$  production experiment (stage III), triplicate sets of glucose control batch reactors were prepared. For the incubation experiments with LA (control) and glucose plus LA, two sets of triplicate batch reactors were prepared and injected with LA ( $2,000 \text{ mg L}^{-1}$ ) (TCI, USA) one day before day 0. Glucose ( $5,000 \text{ mg L}^{-1}$ ) (ACP Chemicals, Montreal, Quebec) was injected on day 0 and again on day 4 in both sets of reactors (glucose control and glucose plus LA). On day 4 following the 1<sup>st</sup> glucose injection, all batch reactors were opened, the pH was re-adjusted to the initial value (pH 5), purged with nitrogen (99.998 %) for 3 minutes and re-sealed again with Teflon lined septa capped with aluminum crimp.

Headspace gases, VFAs, and alcohols were monitored daily for 4 days after each glucose injection for all batch reactors. Glucose degradation was monitored for 24 hrs in glucose control and glucose plus LA samples. Four days after the second glucose injection (Day 8), all serum bottle batch reactors were opened and VSS/TSS measurements were conducted.

#### **3.4.4 Phase IV - Effects of LCFAs shorter than C18 on $\text{H}_2$ production from glucose in mixed culture at $37^{\circ}\text{C}$ and initial pH 5.**

Experiments were designed (Table 3.6) to assess the effects of lauric (LUA), myristic (MA), palmitic (PA), and a mixture of 50:50 myristic:palmitic (MA:PA) [on molar basis] acids on  $\text{H}_2$  production from glucose in anaerobic mixed culture at  $37^{\circ}\text{C}$

and initial pH 5. All serum batch reactors were prepared according to the protocol described in section 3.2.

Table 3.6 Experimental design matrix for the effect of lauric, myristic and palmitic acids on H<sub>2</sub> production with low initial pH.

Sample	Incubation Period (days)	Substrate fed		Liquid and gas Analysis			
		LCFA	Glucose	Glucose	VFAs	Alcohols	H <sub>2</sub> , CH <sub>4</sub> and CO <sub>2</sub>
Glucose control	8	X	√	√	√	√	√
Lauric acid (C12)	8	√	√	√	√	√	√
	8	√	X	X	√	√	√
Myristic acid (C14)	8	√	√	√	√	√	√
	8	√	X	X	√	√	√
Palmitic acid (C16)	8	√	√	√	√	√	√
	8	√	X	X	√	√	√
50:50 mixture of C14:C16	8	√	√	√	√	√	√
	8	√	X	X	√	√	√

A LCFA stock solution (100,000 mg L<sup>-1</sup>) was prepared according to the protocol described in section 3.5.4. The anaerobic cultures utilized in phase IV was a mixture of (80:20 ethanol:municipal Chatham) ratio and maintained according to the protocol described in section 3.1.

Triplicates glucose control batch reactors were prepared to assess the performance of the culture without imposing a stress condition. For incubation experiments with LUA, MA, PA, and 50:50 MA:PA (on molar basis) controls and glucose plus each of LUA, MA, PA, and 50:50 MA:PA, two sets of triplicate batch reactors were prepared and injected with LCFA (7.131 mM) (TCI, USA) one day before day 0. Glucose (5,000 mg L<sup>-1</sup>) (ACP Chemicals, Montreal, Quebec) was injected on day 0 and again on day 4 in both sets (glucose control and glucose plus LCFA). Four days after the first glucose injection (Day 4), all the batch reactors were opened, pH was re-adjusted to the initial value (pH 5), purged with nitrogen (99.998 %) for 3 minutes and re-sealed again with Teflon lined septa capped with aluminum crimp.

Headspace gases, VFAs, and alcohols were monitored daily for 4 days after each glucose injection for all batch reactors. Glucose degradation was monitored for 24 hrs in the glucose control and the glucose plus LCFA samples. Four days after the second glucose injection (Day 8), all the serum bottle batch reactors were opened and VSS/TSS measurements were conducted.

### **3.4.5 Phase V Effects of incubation period, LA degradation by-products, and sludge physical structure on H<sub>2</sub> production from glucose.**

This phase examined the effects of incubation period (0 to 25 days) and LA degradation by-products on H<sub>2</sub> production in flocculated and granular cultures. The flocculated cultures were prepared by passing the original granulated culture through sieve number 200 and then mixing the cultures in a 4 L serum bottle at high rate of approximately 500 rpm for 7 days. Because the cultures originated from the same source, they were assumed to be composed of the same microbial community. Experiments were designed to examine the effects of LA, its degradation by-products, and length of the incubation period on H<sub>2</sub> production in the two cultures (granular and flocculated). The experimental design is summarized in Table 3.7. For each type of culture the following experiments were performed simultaneously.

1. Experiment 1 (LA control): Incubation with LA over a period of 0 to 30 days.
2. Experiment 2: Incubation with LA plus glucose over a period of 0 to 30 days.
3. Experiment 3 (Glucose control): incubation with glucose for every 5 days; conducted concurrently with Experiment 1 (LA control) and Experiment 2 (LA plus glucose) which continued for 30 days.

The maximum incubation period of 25 days was selected because the time frame for the degradation of LCFAs in anaerobic culture is approximately 20 to 25 days (Lalman and Bagley, 2000). This experiment would show whether 2,000 mg L<sup>-1</sup> LA is sufficient to sustain high H<sub>2</sub> production and yield over a relatively long period of time

Table 3.7 Experimental design matrix for studying the effects of incubation period, LA degradation by-products, and culture physical structure on H<sub>2</sub> production from glucose.

glucose:									
Sample	Set No.	Incubation Period (days)	Substrate fed		Liquid and gas Analysis				
			LA (2g L <sup>-1</sup> )	Glucose (5 g L <sup>-1</sup> )	LCFAs	Glucose	VFAs	Alcohols	H <sub>2</sub> , CH <sub>4</sub> and CO <sub>2</sub>
Granular sludge									
LA control		0-30	√	X	√	X	√	√	√
Glucose + LA	Set 1	0-5	√	√	√	√	√	√	√
	Set 2	5-10	√	√	√	X	√	√	√
	Set 3	10-15	√	√	√	X	√	√	√
	Set 4	15-20	√	√	√	X	√	√	√
	Set 5	20-25	√	√	√	X	√	√	√
	Set 6	25-30	√	√	√	X	√	√	√
Glucose control	Set 1	0-5	X	√	√	√	√	√	√
	Set 2	5-10	X	√	√	X	√	√	√
	Set 3	10-15	X	√	√	X	√	√	√
	Set 4	15-20	X	√	√	X	√	√	√
	Set 5	20-25	X	√	√	X	√	√	√
	Set 6	25-30	X	√	√	X	√	√	√
Suspended sludge									
LA control		0-30	√	X	√	X	√	√	√
Glucose + LA	Set 1	0-5	√	√	√	√	√	√	√
	Set 2	5-10	√	√	√	X	√	√	√
	Set 3	10-15	√	√	√	X	√	√	√
	Set 4	15-20	√	√	√	X	√	√	√
	Set 5	20-25	√	√	√	X	√	√	√
	Set 6	25-30	√	√	√	X	√	√	√
Glucose control	Set 1	0-5	X	√	√	√	√	√	√
	Set 2	5-10	X	√	√	X	√	√	√
	Set 3	10-15	X	√	√	X	√	√	√
	Set 4	15-20	X	√	√	X	√	√	√
	Set 5	20-25	X	√	√	X	√	√	√
	Set 6	25-30	X	√	√	X	√	√	√

(one month). Moreover, through the determination of LA degradation by-products it is possible to link H<sub>2</sub> production to a specific by-product at various incubation periods.

The inoculum was maintained in 4-L semi-continuous reactors (37°C) and fed glucose (5 g L<sup>-1</sup>) every 6-7 days. Granular and flocculated cultures were from the same origin (Chatham, ON). Flocculated cultures were produced from the granulated

cultures to examine the effects of LA and its degradation by-products on H<sub>2</sub> production in relation to the physical structure of the culture.

All experiments were conducted in 160 mL serum bottle batch reactors incubated at (37 ± 2°C) with an initial pH of 5 plus VSS 2,000 mg L<sup>-1</sup> of culture. Control experiments with LA were conducted for a period of 30 days. For cultures incubated with LA plus glucose, six triplicate sets (6 incubation periods ranging from 0 to 25 days with an increment of 5 days were adopted) were prepared and injected with LA (2,000 mg L<sup>-1</sup>; TCI, USA), one day before Day 0. Glucose (5,000 mg L<sup>-1</sup>; ACP Chemicals, Montreal, Quebec) was injected on the 0<sup>th</sup>, 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 25<sup>th</sup> days in both sets (glucose control and glucose plus LA). Starting on Day 5, controls were prepared by injecting glucose (i.e. five days before each glucose injections).

Headspace gases, VFAs, and alcohols were monitored daily for 5 days after each glucose injection. LCFAs extraction was performed on Day 5 after the glucose injections (i.e., on the 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, 20<sup>th</sup>, and 25<sup>th</sup> days). Glucose was monitored for set 1 only (day 0). All the serum bottle batch reactors were prepared according to the protocol described in section 3.2.

The objective of LA control experiments was to determine whether LA degradation by-products could sustain H<sub>2</sub> production and to what extent over a period of 30 days. The objectives of the experiment on glucose plus LCFAs were to examine the effects of LA on H<sub>2</sub> production in suspended and granular sludge; to assess the effect of different incubation periods and to assess the effects of LA progenies on the diversion of electron equivalents away from methanogens and other H<sub>2</sub> consuming microorganisms.

### 3.5 ANALYTICAL METHODS

Analytical parameters consisted of liquid (VFAs, glucose, alcohols and LCFAs) and headspace gases ( $H_2$ ,  $CH_4$  and  $CO_2$ ). Liquid samples were purified before they could be analyzed. Procedures for sampling and processing of the VFAs, alcohols and glucose were the same, and were used in all of the experiments. A liquid extraction method was adopted and modified for LCFA recovery using MTBE: Hexane:chloroform (1:1:1) (Lalman and Bagley, 2000) as described in section 3.5.4.

#### 3.5.1 VFA Measurements

At selected time intervals, samples were withdrawn using a 0.25 to 0.5 mL Hamilton Gastight (VWR, Canada) syringe, 0.5 mL samples were transferred into 10 mL culture tubes containing 4.5 mL of Milli-Q (Millipore, Barnstead, USA) grade water. The 10 to 20 times diluted samples were centrifuged at 1750 g for 5 minutes to remove biological and inorganic solids. The centrate was filtered using 2 filters connected in series to remove suspended solids and heavy metals, respectively. The first filter consisted of a 25 mm diameter syringe filter holder (PAL Sciences, MI, USA) fitted with a 25 mm diameter 0.45  $\mu$ m polypropylene membrane (GE Osmonics, MN) and the second filter was a 1 mL polypropylene cartridge tube fitted with a 20  $\mu$ m polyethylene frit (Spe-ed Accessories, PA) and filled with 1 gm of 100 to 200 mesh ion exchange sodium resin (Chelex 100, Bio-Rad Laboratories, CA). The filtrate was transferred to a 5.0 mL polypropylene vial (Dionex, Oakville, ON) and stored at 4°C prior to analysis.

The filtered samples were analyzed for VFAs using a Dionex DX-500 ion chromatograph (IC) (Oakville, ON) equipped with a CD 20 conductivity detector, ASRS suppressor, a GP 40 gradient pump, an AS 40 automated sampler and a 25- $\mu$ L-sample loop. The IC was configured with a Dionex IonPac 24 cm x 4 mm diameter



AS11-HC analytical column and a Dionex IonPac AG11-HC guard column. VFAs in phase III experiments were analyzed using the following three eluents: 1) Eluent A, Milli-Q grade water (18 M $\Omega$ .cm); 2) Eluent B, 100 mM NaOH; and 3) Eluent C, 1 mM NaOH. The total eluent flow rate was 2 mL min<sup>-1</sup> and the individual flow rates as percentage of the total flow were: 0 - 15 min, 80 % A, 20 % C; 15 - 15.1 min, 85 % A, 15% B; 0 % C ; 15.1 - 25 min, 65 % A, 35 % B ( Lalman and Bagley, 2000). The detection limit (including dilution) for formate, acetate, propionate and butyrate was 0.5 mg L<sup>-1</sup>.

Triplicate standards of 0 (Milli-Q water), 0.5, 1, 2, 3, 4, 5, 10, 30, 50, 70 and 100 mg L<sup>-1</sup> for lactate, acetate, propionate, butyrate and formate were prepared from 5,000 mg L<sup>-1</sup> stock solutions and used for instrument calibrations. The VFAs calibration curve plots are shown in Appendix A. During sample analysis, intermediate standards were analyzed after every 10 samples. A variation of  $\pm 5$  % from the calibration standards was considered acceptable. The detection limits for all the VFAs was 0.5 mg L<sup>-1</sup>.

### **3.5.2 Glucose and Alcohol Measurements**

For glucose analysis, the samples were analyzed using a Dionex-600 ion chromatograph (IC) equipped with a GP 50 gradient pump, an AS 40 automated sampler, an ED 50 electrochemical detector and a 25- $\mu$ L-sample loop (Lalman *et al.*, 2002). The instrument was configured with a Dionex CarboPac™ 25 cm x 4 mm diameter MA1 analytical column (Dionex, Sunnyvale, CA), and a Dionex CarboPac™ 5 cm x 4 mm diameter MA1 guard column (Dionex, Sunnyvale, CA). Some glucose samples were analyzed using a 25-cm  $\times$  4-mm CarboPac™ PA-20 column (Dionex, Sunnyvale, CA). The isocratic method used a 480 mM NaOH eluent with a flow rate set at 0.4 mL min<sup>-1</sup> for the MA-1 column and 20 mM NaOH at

a flow rate  $0.2 \text{ mL min}^{-1}$  for the PA-20 column. The detection limit for glucose on both columns was  $1.0 \text{ mg L}^{-1}$ . Total analysis run time was 35 minutes; glucose was detected at approximately 25 minutes, and ethanol, propanol, i-propanol, butanol, and i-butanol were detected at 7.38, 7.68, 8.98, 10.46 and 11.53 minutes, respectively.

Triplicate standards of 0 (Milli-Q water), 1, 2, 3, 4, 5, 10, 50, 100, 200, 300, 400, and  $500 \text{ mg L}^{-1}$  for glucose were prepared from  $100,000 \text{ mg L}^{-1}$  stock solutions by serial dilution and used for instrument calibrations. The glucose calibration curve plots are shown in Appendix A. During sample analysis, intermediate standards were analyzed after every 10 samples. A variation of  $\pm 5 \%$  from the calibration standards was considered acceptable. The detection limits for glucose was  $1.0 \text{ mg L}^{-1}$ .

Triplicate standards of 0 (Milli-Q water), 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 75, and  $100 \text{ mg L}^{-1}$  of ethanol, propanol, i-propanol, butanol, and i-butanol alcohols were prepared from  $5,000 \text{ mg L}^{-1}$  stock solutions and used for instrument calibration. The calibration curve plots for the alcohols are shown in Appendix A. During sample analysis, intermediate standards were analyzed after every 10 samples. A variation of  $\pm 5 \%$  from the calibration standards was considered acceptable. The detection limits for all alcohols was  $5.0 \text{ mg L}^{-1}$ .

### **3.5.3 Headspace Gas Analysis**

The headspace gas samples ( $25 \mu\text{L}$ ) were manually injected into a Varian 3800 gas chromatograph (GC) (Varian, Palo Alto, CA) using a  $50 \mu\text{L}$  Hamilton Gastight syringe (VWR, Canada). The GC was configured with a thermal conductivity detector (TCD). and a  $2 \text{ m} \times 1 \text{ mm}$  diameter (ID) packed Shincarbon ST (Restek) column. The analysis was isothermal with the oven temperature set at  $200^{\circ}\text{C}$ . The detector and injector temperatures were  $200^{\circ}\text{C}$  and  $100^{\circ}\text{C}$ , respectively. The nitrogen (99.999%, Praxair, ON) carrier gas flow was set at  $21 \text{ mL min}^{-1}$ . The detection limits for

hydrogen, methane and carbon dioxide were 0.1900 kPa (2.5  $\mu$ L/bottle), 0.1226 kPa (2.5  $\mu$ L/bottle) and 0.0737 kPa (2.5  $\mu$ L/bottle), respectively.

Calibration standards for the gas chromatograph were prepared in serum bottles (160 mL). The bottles were purged with nitrogen (99.998 %) for 3.0 minutes and capped with aluminum crimp sealed Teflon-lined septa. Known quantities of H<sub>2</sub>, CH<sub>4</sub> and CO<sub>2</sub> were injected into the capped bottles and analyzed. During each experimental headspace analysis, standards were prepared within the calibration range and analyzed for the different gases. The gas calibration curves are shown in Appendix A.

#### **3.5.4 LCFAs Measurements**

The extraction method was based on the method described by Lalman and Bagley (2000) with a slight modification to ensure accurate measurement of the aqueous samples. 1.0 mL samples were withdrawn from the serum bottles for LCFA analysis. The 1.0 mL sample was placed in a 5 mL vial containing 3 mL of 1:1 Hexane:MTBE:chloroform (EM Science, USA), 0.05 g NaCl (ACP Chemicals, Montreal, Quebec), and 2 drops of 50% H<sub>2</sub>SO<sub>4</sub> (EMD Chemicals, USA). The vial was sealed with Teflon<sup>R</sup> lined septa and capped with aluminum crimp seals and placed on an orbital shaker at 37°C (Lab Line Instruments, USA; Model HHKE 4000) for 30 min at 200 rpm. Next, the vial was centrifuged for 5 min at 1750 g. The organic phase was removed and 1.0  $\mu$ L of the sample was analyzed using a Varian 3800 GC equipped with a flame ionization detector (FID) and a split/splitless injector. LCFAs (C6:0 to C18:2) were separated on a DB-FFAP 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m analytical column (J and W Scientific, USA). The injector and the FID were maintained at 240°C and 250°C, respectively. The oven temperature was programmed as follows: 1) 100°C for 2.0 minutes; 2) temperature was increased to 240°C in 15°C.min<sup>-1</sup>

gradients; and 3) held at 240°C for 8.67 minutes. The total analysis time was 20 minutes. Helium was the carrier gas at a constant pressure of 30 psi; split was off for 0.01 min, then on at a split ratio of 70:1 until the end of the run.

Prior to LCFA sample analysis, LCFA extraction studies were conducted to establish the percentage recovery. Recovery studies were conducted with C6 to C18:2 LCFAs; caprylic acid (C8:0, >99%), capric acid (C10:0, >99%), lauric acid (C12:0, >99%), myristic acid (C14:0, >99%), palmitic acid (C16:0, >99%), stearic acid (C18:0, >99%), oleic acid (C18:1) and linoleic acid (C18:2, >99%) (TCI, USA). Extraction studies were performed using 10, 50, 100, 300, 500, 1000, and 1500 mg L<sup>-1</sup> of all the LCFAs in basal medium at 37°C. The required concentrations of the LCFA mixture were injected from a 20,000 mg L<sup>-1</sup> LCFAs stock solution which was prepared using the saponification technique of LCFA melted *au bain-marie* in hot NaOH (Rinzema *et al.*, 1994). The amount of NaOH added is shown in Table 3.8. The percentage recovery in all of the extraction studies at all of the concentrations ranged between 85-100 %. Plots of percentage recovery for C6 to C18:2 LCFAs are shown in Appendix A. Calibration curves for LCFAs were prepared in hexane from a 5,000 mg L<sup>-1</sup> stock solution, and are shown in Appendix A. The detection limits were 5 mg L<sup>-1</sup> for caprylic (C8:0) and palmitic (C16:0) acids and 15 mg L<sup>-1</sup> for stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids.

Table 3.8 Quantity of sodium hydroxide used for LCFA stock solution preparation.

LCFA	NaOH (g g <sup>-1</sup> LCFA)
Caprylic	0.278
Capric	0.233
Lauric	0.200
Myristic	0.175
Palmitic	0.156
Stearic	0.141
Oleic	0.142
Linoleic	0.143

### 3.5.4.1 Optimization of LCFAs extraction method

The method for LCFAs extraction used in this work was based on the method described by Lalman and Bagley (2004). A study was conducted to optimize the method for the recovery of high concentrations (up to 2,000 mg L<sup>-1</sup>) of individual LCFAs from the fermentation broth as solvent type and concentrations were problematic with the original method. Lalman and Bagley (2004) used a mixture of 1:1 (volume) of Hexane:Methyl tertiary butyl ether (H:MTBE). This combination of solvents did not recover all of the high concentrations of individual LCFAs from the fermentation broth. The efficiency of the extraction (expressed as percent recovery of C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, and C18:2 when a mixture of 1:1 (H:MTBE) was used) is shown in Figure 3.1(A). The percent recovery for concentrations of 500 mg L<sup>-1</sup> of the individual LCFAs was equal to or less than 80-85% for C6 to C12, and less than 60% for C14 to C18:2. Chloroform was examined as an alternative solvent. The same extraction protocol was followed with 1 volume of chloroform instead of 1:1 H:MTBE. Chloroform solved the problem of recovering high concentrations of the individual LCFAs longer than C10:0, but was not sufficient to recover the individual LCFAs shorter than C12 (Figure 3.1(B)). To ensure that both longer and shorter LCFAs were recovered with high efficiency at both low and high concentrations, a mixture of 1:1:1 H:MTBE:Chloroform (volume) was used. The recovery efficiency for C6 to C18:2 is shown in Figure 3.1(C). The recovery percentages for 10 to 2,000 mg L<sup>-1</sup> of C6, C8, C10, C12, C14, C16, C18:0, C18:1, and C18:2 are shown in Figure 3.2; recovery of C10, C12, C14, C16, C18:0, and C18:2 was greater than 95% .

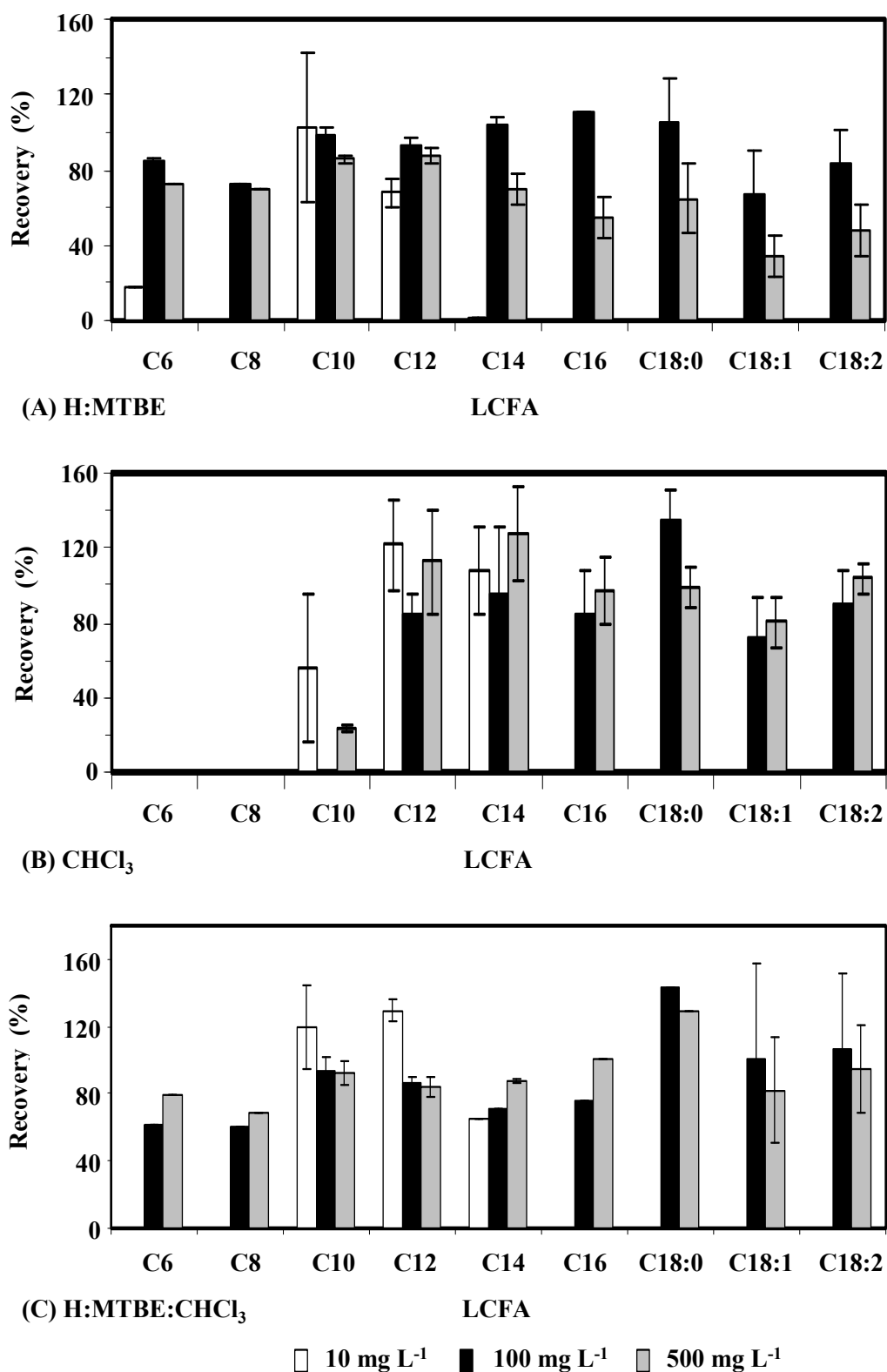


Figure 3.1 Recovery efficiency of LCFA from fermentation broth using (A) 1:1 volume ratio mixture of H:MTBE, (B) 1 volume of CHCl<sub>3</sub>, and (C) 1:1:1 volume ratio mixture of H:MTBE:CHCl<sub>3</sub>. (Averages and standard deviations of triplicate samples are shown).

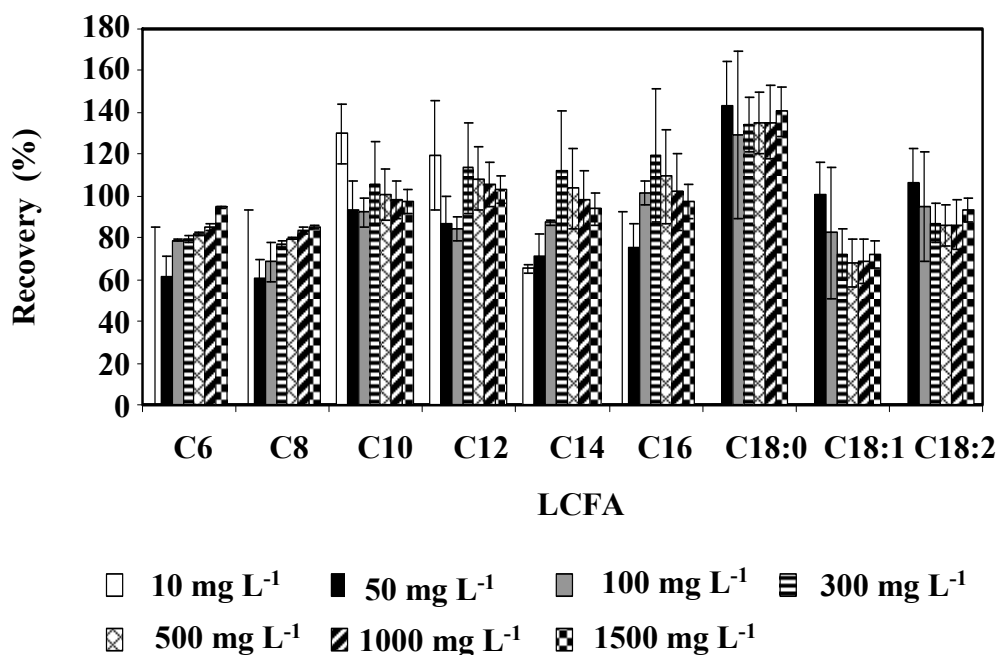


Figure 3.2 Recovery efficiency of C6:0 to C18:2 using 1:1:1 volume ratio mixture of Hexane:Methyl tertiary butyl ether:Chloroform. (Averages and standards deviations of triplicate samples are shown).

### 3.5.5 pH and Measurement of Total Solids

The pH of each batch reactor was measured at the beginning and at the end of each experiment using a VWR SR40C, Symphony pH meter. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined at the beginning of each experiment. The analysis was conducted using 5 mL of liquid sample and 1- $\mu$ m pore size glass fiber filter papers (VWR, Canada). TSS, VSS, and pH were determined according to the procedures described in Standard Methods (APHA *et al.*, 1998).

## 3.6 TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

### 3.6.1 Genomic DNA extraction

A sludge sample (0.4 mL) was added to a 2 mL tube containing approximately 250 mg of zirconia/silica beads, 0.4 mL cetyl trimethylammonium bromide (CTAB) extraction buffer (20% (wt/vol) CTAB (Sigma, Toronto, ON) in 1.4 M NaCl with 480 mM potassium phosphate buffer at pH 8.0 and 0.4 mL of phenol–chloroform–isoamyl

alcohol (25:24:1; pH 8.0). The mixture was subjected to three freeze (-80°C) and thaw cycles. Bacteria cells in the sample were lysed by homogenizing for 45s in a Thermo Savant Bio 101/FP120 Fast prep homogenizer at a 6.5 speed. Phase separation was achieved by centrifugation 16,000×g for 10 min. at 4°C. The clear aqueous upper phase was transferred to micro-centrifuge tubes and re-extracted by mixing with an equal volume of chloroform–isoamyl alcohol (24:1). This was followed by centrifugation (10,000×g) for 10 min. Nucleic acids were then precipitated from the extracted aqueous layer with 0.6 vol of isopropanol for 10 min. at room temperature. The sample was then centrifuged (10,000×g) at 5°C for 20 min. Nucleic acids pellets were washed in 70% (v/v) ice-cold ethanol and air dried before re-suspension in 50 µL sterile MilliQ water.

### **3.6.2 PCR and T-RFLP profiling**

Nested-PCR of the microbial community 16S rRNA gene was performed using dye-labeled primers B8F (5’-/AGAGTTTGATCCTGGCTCAG-3’) (Edwards *et al.*, 1989) with reverse primers Eub-539R (5’- ATCGTATTACCGCGGCTGCTGGC-3’). Similarly, archaeal 16S rRNA genes were amplified with primers Arc-112F (5’-/GCTCAGTAACACGTGG-3’) and Arc-533R (5’-TTACCGCGGCGGCTGGCA-3’) reverse primer. PCR mixtures (25 µL) containing 10.2 mM Tris buffer, 2.3 mM MgCl<sub>2</sub>, 50 mM KCl, 2% DMSO, 5 mg BSA, 0.2 mM of each dNTP, 0.2 mM of each primer, and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Streetsville, ON) were cycled as follows: 95°C for 5 min; 35 cycles of 94°C for 1 min, 42°C for 30s, 72°C for 30s and subsequently with a final elongation step of 72°C for 1 min. Purified PCR products (using QIAquick spin columns, Invitrogen, 3 µL) were digested with 2.5 U of restriction enzyme (*Hae* III *Hha* I, *MSP*-I and *Hinf* I) in a total volume of 20 µL for 2 h at 37°C. *Hae* III and *Hha* I were selected because they



produce several short terminal restriction fragments (T-RFs) (<300 bases) from most bacteria and archaea. The restriction digested products (1  $\mu$ L) were mixed with 3  $\mu$ L of stop solution (LI-COR, Inc., Lincoln, NE) and samples along with the size markers (50-700 base pairs, LI-COR, Inc., Lincoln, NE) were denatured at 95°C for 2 min. and then rapidly chilled with ice. The denatured samples were loaded onto a 6.5% polyacrylamide gel (KBPlus™, LI-Cor, Inc., Lincoln, NE) and separated by size by electrophoresis (2.5 h at 1500 V, 35 mA, 35 W, 45°C) using a DNA analyzer (Model 4300L, LI-Cor, Inc., Lincoln, NE).

### **3.6.3 Statistical analysis of T-RFLP patterns**

The software Gene ImagIR 4.05 (Scanlytics, Inc.) was used to estimate the fragment sizes and relative intensity. T-RFs that differed by  $\pm 1$  bp in different profiles were considered as identical in order to compare the T-RFLP profiles between the samples. The relative intensity of each T-RF within a given T-RFLP pattern was calculated as the peak area of the respective T-RF divided by the total peak area of all T-RFs detected within a fragment length range between 40 and 600 bp for archaea and bacteria. Fragments smaller than 40 bp were excluded from the analysis.

### **3.6.4 Phylogenetic assignment of T-RFs**

T-RFLP data generated by digestion of sludge DNA samples with restriction enzymes (*Hae* III and *Hha* I) were formatted according to Phylogenetic Assignment Tool (PAT) requirements and analyzed on-line using default fragment bin tolerance window setting (<https://secure.limnology.wisc.edu/trflp/>). Phylogenetic assignment was performed using a modified database consisting of the default database generated from microbial community analysis (MiCA).

### 3.7 FLUX BALANCE ANALYSIS

The flux balance analysis (FBA) was applied using data gathered from phase I, III, IV, and V experiments. The flux analysis was applied to experimental data using the metabolic reactions network with stoichiometries described in the literature. The MFA was carried out using the MetaFluxNet software, Version.1.8.6.2 (URL: <http://mbel.kaist.ac.kr/mfn>). The metabolic reactions network was based on the model developed by Chaganti *et al.* (2011) and shown in Figure 3.3.

The stoichiometries of the reactions (R1 to R30) are given in Table 3.9. The dashed line in Figure 3.3 represents the cell wall and distinguishes the intercellular and extracellular metabolites. The input included the external substrate (glucose) and the measured by-products (gases, VFAs, and alcohols). Acetate or acetone production was selected as the objective functions.

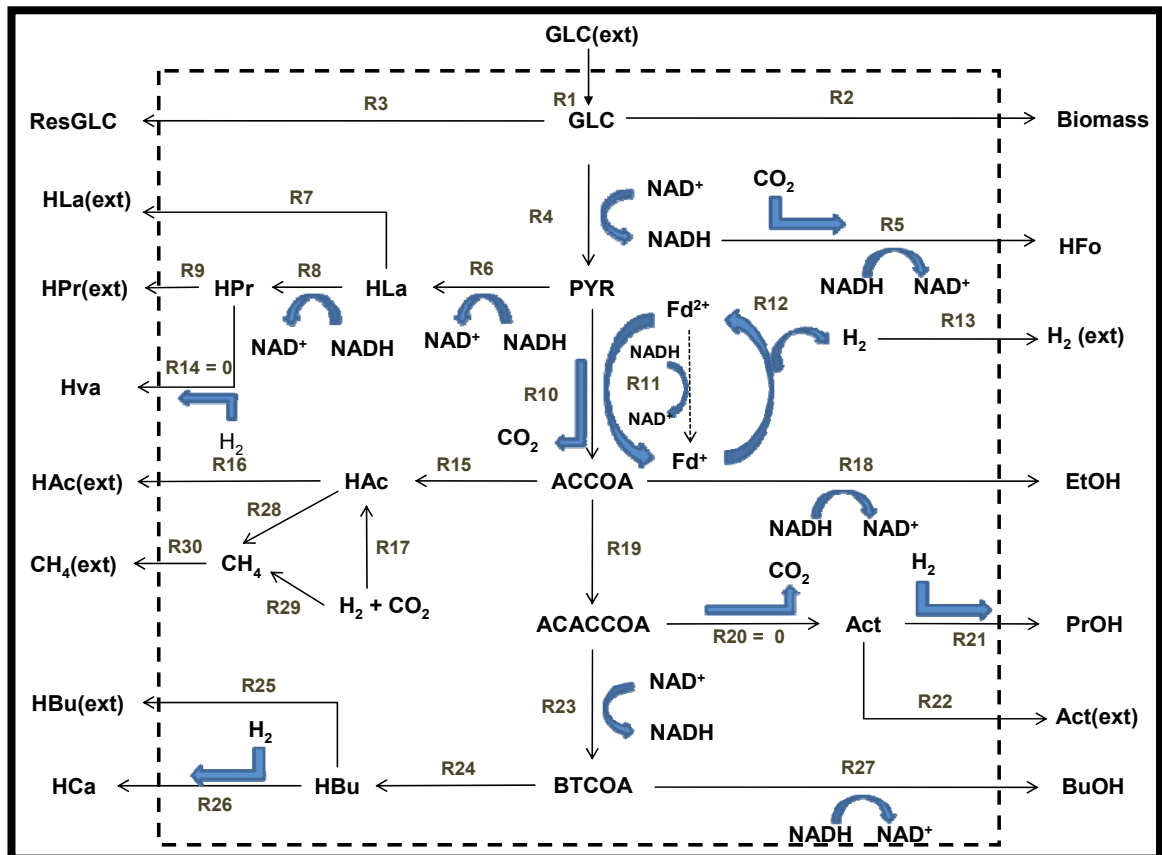


Figure 3.3 Metabolic reaction network of the model used in metabolic flux analysis. (Adapted from Chaganti *et al.*, 2011).

Table 3.9 Stoichiometries of the bioreactions (R1 to R30) in the metabolic reaction network of the model used in metabolic flux analysis.

Reaction	Reaction stoichiometry
R1	Glucose(ext) $\rightarrow$ Glucose
R2	Glucose $\rightarrow$ Biomass
R3	Glucose $\rightarrow$ Residual glucose
R4	Glucose $\rightarrow$ 2 Pyruvate + 2 NADH
R5	NADH $\rightarrow$ H <sub>Fo</sub>
R6	NADH + Pyruvate $\rightarrow$ HLa
R7	HLa $\rightarrow$ HLa(ext)
R8	HLa + NADH $\rightarrow$ HPr
R9	HPr $\rightarrow$ HPr(ext)
R10	Pyruvate $\rightarrow$ AcetylCoA + 2 Fdred
R11	NADH $\rightarrow$ 2 Fdred
R12	2 Fdred $\rightarrow$ H <sub>2</sub>
R13	H <sub>2</sub> $\rightarrow$ H <sub>2</sub> (ext)
R14	HPr + 6 H <sub>2</sub> $\rightarrow$ HVa
R15	AcetylCoA $\rightarrow$ HAc
R16	HAc $\rightarrow$ HAc(ext)
R17	4 H <sub>2</sub> $\rightarrow$ HAc
R18	AcetylCoA + 2 NADH $\rightarrow$ EtOH
R19	2 AcetylCoA $\rightarrow$ AcetoacetylCoA
R20	AcetoacetylCoA $\rightarrow$ Acetone
R21	Acetone + H <sub>2</sub> $\rightarrow$ PrOH
R22	Acetone $\rightarrow$ Acetone(ext)
R23	AcetoacetylCoA + 2 NADH $\rightarrow$ ButyrylCoA
R24	ButyrylCoA $\rightarrow$ H <sub>Bu</sub>
R25	H <sub>Bu</sub> $\rightarrow$ H <sub>Bu</sub> (ext)
R26	H <sub>Bu</sub> + 6 H <sub>2</sub> $\rightarrow$ H <sub>Ca</sub>
R27	ButyrylCoA + NADH $\rightarrow$ BuOH
R28	HAc $\rightarrow$ CH <sub>4</sub>
R29	4 H <sub>2</sub> $\rightarrow$ CH <sub>4</sub>
R30	CH <sub>4</sub> $\rightarrow$ CH <sub>4</sub> (ext)

### 3.8 PRINCIPAL COMPONENTS ANALYSIS and STATISTICAL TESTS

Principal components analysis was applied using STATISTICA<sup>®</sup> data analysis software, Version 7.0.61.0 (StatSoft, Inc., 2004; [www.statsoft.com](http://www.statsoft.com)). Other statistical significance comparison tests (two tailed t-test and Tukey's multiple comparison test) were carried out using Microsoft Excel 2003 (Box *et al.*, 1978).

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## **CHAPTER 4: EFFECTS OF CULTURE SOURCE ON BIOHYDROGEN PRODUCTION IN THE PRESENCE OF LINOLEIC ACID**

### **4.1 INTRODUCTION**

The era of the long dependency on non-renewable fossil fuels is approaching its end. Life expectancy for oil is approximately 50 years, hence researchers are forging ahead to develop alternative sustainable energy supplies. Biomass residues offer renewable resources for bioenergy, however, exploitation methods need to be developed and optimized. Hydrogen ( $H_2$ ) is considered the next energy carrier because of its high energy content, and releasing non-polluting combustion by-products. Hydrogen could be produced by several methods; however, biological routes are the cheapest. Of the biological  $H_2$  production methods, dark fermentation using mixed anaerobic cultures offers the most competitive continuous production because it does not need any energy input and could use waste as a substrate.

The bioprocess of  $H_2$  production via dark fermentation using mixed cultures depends on the composition, the relative populations, and activity of their microbial communities (Harper and Pohland, 1986). The nature and influence of the seed culture used for  $H_2$  production should be accounted for, because changes in the bacterial populations may alter how they function (Guyot *et al.*, 1993; Wittebolle *et al.* 2005; Temudo *et al.*, 2008). For example, the ability of the culture to metabolize various substrates such as carbohydrates, or “intermediate” products of anaerobic metabolism such as lactate, propionate, butyrate, and acetate depends strongly on the particular mixture of bacterial species (Gavala and Lyberatos, 2001, Karsubasia *et al.*, 2005). Differences in the sources

of the inocula can affect the amount of H<sub>2</sub> produced (Danko *et al.*, 2008a, 2008b; Lay *et al.*, 1999; Li and Fang, 2007; Shin *et al.*, 2004; Valdez-Vazquez *et al.*, 2006; van Ginkel *et al.*, 2001; Yu *et al.*, 2002). Therefore, selection of the appropriate culture will obviously lead to a better performance (Demirel and Scherer, 2008).

With diverse microbial communities present in mixed cultures from various sources, the selection of the culture is not an easy task. The first step is to characterize different anaerobic cultures in H<sub>2</sub> production studies under various stress conditions. Danko *et al.* (2008a) reported a difference in H<sub>2</sub> production between suspended cultures from a municipal anaerobic digester and a granular cultures from a brewery wastewater treatment facility. These researchers attributed the differences in H<sub>2</sub> production and behavior of the two sludges to differences in their microbial communities. Suspended and granular cultures from municipal and industrial wastewater treatment facilities reportedly differ in H<sub>2</sub> production from arabinose; for example, suspended sludge gave a higher H<sub>2</sub> yield than granular cultures at pH 7, 6, and 5 (Table 4.1) (Abreu *et al.*, 2009). Wang *et al.* (2007) reported that a UASB reactor seeded with a methanogenic culture and operated at low pH had a higher yield, production rate, and partial pressure of H<sub>2</sub> than a USAB reactor seeded with heat-treated cultures. According to Akutsu *et al.* (2008), differences in H<sub>2</sub> production from five different types of cultures were related to differences in the microbial community structures.

Mixed anaerobic cultures contain both H<sub>2</sub>-producing and H<sub>2</sub>-consuming microorganisms. Studies carried out with various substrates have shown that the methods used to

Table 4.1 H<sub>2</sub> yield from different types of cultures.

Sludge type	Substrate	Pretreatment	pH	H <sub>2</sub> yield <sup>1</sup>	Reference
Granular brewery	Arabinose	Heat	6.5	1.3	Abreu <i>et al.</i> , 2009
			5	0.8	
Suspended municipal	Arabinose	Heat	7.0	2.0	
			6.0	2.5	
			5.0	1.5	
Digested activated sludge	Glucose	No	4.9	2.32	Akutsu <i>et al.</i> , 2008
Digested cattle manure	Glucose	No	5.4	1.71	
Compost of NS & OFMSW <sup>2</sup>	Glucose	No	5.3	2.13	
Acidified potato	Glucose	No	4.9	2.02	
Digested NS & OFMSW	Glucose	No	5.4	1.38	
Methanogenic culture	Glucose	No	4.0	1.51	Wang <i>et al.</i> , 2007
Methanogenic culture	Glucose	Heat	4.0	1.19	

<sup>1</sup> In mol H<sub>2</sub>•mol<sup>-1</sup> substrate.

<sup>2</sup> NS & OFMSW = night soil and organic fraction of municipal waste.

inhibit methanogens such as 2-bromoethanesulfonic acid (BESA), heat treatment, and acid/base treatment can affect H<sub>2</sub> production negatively (Cheong and Hansen, 2006; Kraemer Bagley, 2007; Oh *et al.*, 2003; Valdez-Vazquez *et al.*, 2006; Zhu and Beland, 2006). Inhibiting methanogenesis alone does not guarantee maximum H<sub>2</sub> production and yield if reducing equivalents are not directed towards H<sub>2</sub>. Inefficient relocation of electrons spared from methanogenesis decreases H<sub>2</sub> production and increases other by-products. In comparison to BESA and heat treatment, long chain fatty acids (LCFAs) were shown to enhance H<sub>2</sub> yield from carbohydrate-based substrate in mixed cultures (Ray *et al.*, 2008; Chowdhury *et al.*, 2007). LCFAs are able to control the flow of electron flux and divert them towards H<sub>2</sub> instead of other by-products under inhibited methanogenesis (Ray *et al.*, 2009).

The physical characteristics of the culture determine its susceptibility to inhibition by LCFAs. Various sludges responded differently to the toxicity of LCFAs depending on their specific surface area and size distribution (Hwu *et al.*, 1996).

To what extent the culture source affects the microbial community and the H<sub>2</sub> production potential at low pH in the presence of LCFA is an important question. The objective of this study was to examine the effect of linoleic acid (LA, C18:2) on H<sub>2</sub> production from three different microbial cultures (two industrial methanogenic cultures and one municipal anaerobic culture) under mesophilic and low pH conditions. Improvements in the understanding changes in the populations and microbial processes during dark fermentation H<sub>2</sub> production are crucial for better design and control.

## **4.2 MATERIALS AND METHODS**

The sources of the inocula and maintenance of the cultures used in this work are described in section 3.1. Details of the experimental design are described in section 3.4.1. The analytical methods used are described in section 3.5. All experiments were carried out at 37±2°C with an initial pH of 5.

## **4.3 RESULTS**

### **4.3.1 Hydrogen production**

The yield of H<sub>2</sub> from the various sludges examined showed a wide variability as did the response of the various cultures to the inhibition exerted by LA. Peak H<sub>2</sub> yields of 3.13±0.14, 3.11±0.17, and 3.11±0.02 mol H<sub>2</sub>·mol<sup>-1</sup> glucose were obtained from cultures CLA, BLA, and ALA (cultures treated with LA) respectively, whereas, in the control



cultures (CC, BC, and AC) the H<sub>2</sub> yields were 2.80±0.12, 2.80±0.48, and 1.61±0.12 mol H<sub>2</sub>·mol<sup>-1</sup> glucose, respectively (Table 4.2).

Table 4.2 Hydrogen yield in cultures A, B, and C from 5,000 mg L<sup>-1</sup> glucose fermentation without and with 2,000 mg L<sup>-1</sup> LA (glucose added at day 0 and day 5; mean± standard deviations for triplicate samples is shown).

Hydrogen yield (mol H <sub>2</sub> ·mol <sup>-1</sup> glucose)						
Day	Culture A		Culture B		Culture C	
	AC	ALA	BC	BLA	CC	CLA
1	0.27±0.07 <sup>a</sup>	0.65±0.19 <sup>b</sup>	0.07±0.02 <sup>a</sup>	0.43±0.19 <sup>b</sup>	1.20±0.11 <sup>a</sup>	0.69±0.13 <sup>b</sup>
2	1.20±0.09 <sup>a</sup>	0.70±0.18 <sup>b</sup>	<b>0.80±0.11<sup>a</sup></b>	<b>0.93±0.01<sup>a</sup></b>	<b>1.85±0.11<sup>a</sup></b>	<b>0.83±0.16<sup>b</sup></b>
3	1.39±0.05 <sup>a</sup>	1.17±0.29 <sup>a</sup>	0.69±0.10 <sup>a</sup>	0.87±0.24 <sup>a</sup>	1.79±0.07 <sup>a</sup>	0.78±0.14 <sup>b</sup>
4	1.56±0.12 <sup>a</sup>	<b>1.22±0.31<sup>a</sup></b>	0.54±0.16 <sup>a</sup>	0.76±0.29 <sup>a</sup>	1.73±0.07 <sup>a</sup>	0.74±0.13 <sup>b</sup>
5	<b>1.61±0.12<sup>a</sup></b>	1.17±0.32 <sup>a</sup>	0.45±0.18 <sup>a</sup>	0.69±0.30 <sup>a</sup>	1.65±0.08 <sup>a</sup>	0.76±0.10 <sup>b</sup>
6	0.02±0.00 <sup>a</sup>	2.18±0.92 <sup>b</sup>	2.67±0.05 <sup>a</sup>	2.69±0.51 <sup>a</sup>	2.68±0.20 <sup>a</sup>	2.44±0.47 <sup>a</sup>
7	0.03±0.01 <sup>a</sup>	2.73±0.19 <sup>b</sup>	2.69±0.05 <sup>a</sup>	<b>3.11±0.07<sup>b</sup></b>	2.78±0.06 <sup>a</sup>	<b>3.13±0.14<sup>b</sup></b>
8	0.92±0.03 <sup>a</sup>	2.97±0.17 <sup>b</sup>	<b>2.80±0.48<sup>a</sup></b>	2.94±0.09 <sup>a</sup>	<b>2.80±0.12<sup>a</sup></b>	3.00±0.25 <sup>a</sup>
9	1.14±0.04 <sup>a</sup>	<b>3.11±0.02<sup>b</sup></b>	2.43±0.08 <sup>a</sup>	2.67±0.23 <sup>a</sup>	2.72±0.11 <sup>a</sup>	2.71±0.10 <sup>a</sup>
10	<b>1.17±0.08<sup>a</sup></b>	2.18±0.92 <sup>a</sup>	2.35±0.12 <sup>a</sup>	2.63±0.00 <sup>a</sup>	2.49±0.09 <sup>a</sup>	2.61±0.08 <sup>a</sup>

Notes: 1-Control and LA treated samples of the same sludge with the different superscripts (a and b) are significantly different, (p < 0.05). 2-Numbers in bold font indicate the maximum yields after the first and the second glucose injections.

Within one day after the second glucose injection, 90% and 85% of the peak H<sub>2</sub> yields were produced in cultures BLA and CLA respectively, whereas, only 70% of the maximum H<sub>2</sub> yield was produced in culture ALA.

LA decreased the lag phase in H<sub>2</sub> production for all cultures after the second glucose injection. In comparison to cultures A and B, culture C (with and without LA) showed no lag phase in H<sub>2</sub> production after both the first and second glucose injections. Culture B did not show any lag phase after the second glucose injection. After the first glucose injection, culture CC and AC produced more H<sub>2</sub> than culture CLA and ALA.

The response of culture C to the LA effect was contrary to what was expected. Culture C produced more H<sub>2</sub> without LA than with LA after the first glucose injection and almost

the same quantity of H<sub>2</sub> after the second glucose injection. Culture C seemed relatively insensitive to LA after the second glucose injection (Table 4.2). Culture B also produced a high yield of H<sub>2</sub> (3.11 mol H<sub>2</sub>·mol<sup>-1</sup> glucose) on day 7, but the effect of LA was very obvious.

Generally, LA increased the H<sub>2</sub> production rate in all cultures except C (Table 4.3), which showed a lower H<sub>2</sub> production rate in the presence of LA (23.07±4.81 and 141.1±29.82  $\mu\text{mol H}_2 \text{ h}^{-1}$ ) in comparison to the control (110.7±1.10 and 156.9±13.28  $\mu\text{mol H}_2 \text{ h}^{-1}$ ) after the first and second glucose injections, respectively. The maximum H<sub>2</sub> production rate of 156.9±13.28  $\mu\text{mol H}_2 \text{ h}^{-1}$  was observed in culture C control sample after the second glucose injection.

Table 4.3 Maximum hydrogen production rates after glucose injections (on Day 0 and Day 5). (Mean±standard deviations of triplicate samples are shown).

<b>Glucose injection no.</b>	<b><u>Hydrogen production rate (<math>\mu\text{mol H}_2 \text{ h}^{-1}</math>)</u></b>		
	<b><u>Culture</u></b>	<b><u>Control</u></b>	<b><u>LA-treated</u></b>
<b>First</b>	<b>A</b>	34.09±1.32 <sup>a</sup>	31.15±10.78 <sup>a</sup>
	<b>B</b>	47.04±10.21 <sup>a</sup>	50.00±1.53 <sup>a</sup>
	<b>C</b>	110.7±1.10 <sup>a</sup>	23.07±4.81 <sup>b</sup>
<b>Second</b>	<b>A</b>	16.11±13.56 <sup>a</sup>	42.39±23.69 <sup>a</sup>
	<b>B</b>	107.3±75.53 <sup>a</sup>	154.9±35.68 <sup>a</sup>
	<b>C</b>	156.9±13.28 <sup>a</sup>	141.1±29.82 <sup>a</sup>

Note: Values in the same row with the different superscripts (a and b) are statistically different with respect to the controls ( $p < 0.05$ ). Values within the same column were not compared.

### 4.3.2 Methane production

Methane was observed in all control and LA-inoculated cultures after the first glucose injection with a maximum of approximately 600  $\mu\text{mol CH}_4/\text{bottle}$  on Day 4 in control

samples from culture B. Methane production was inhibited in LA-treated cultures after the second glucose injection (Figure 4.1); a maximum of approximately 30  $\mu\text{mol CH}_4/\text{bottle}$  was observed in BLA samples after the second glucose injection.

In terms of electrons diverted to methane, culture CC channelized 3.0% and 4.0% of the electrons available in glucose to methane (after the first and second glucose injections, respectively) compared to only 1.0% and 0.2% in culture CLA. Similarly, the proportion of electrons consumed in methane production in culture BC and AC were 14.0% and 4.0% after the first glucose injection, and 9.0% and 2.3% after the second glucose injection; whereas, for BLA and ALA the percentages were 11.0% and 4.0% after the first glucose injection and 7.0% and 2.0% after the second glucose injection, respectively.

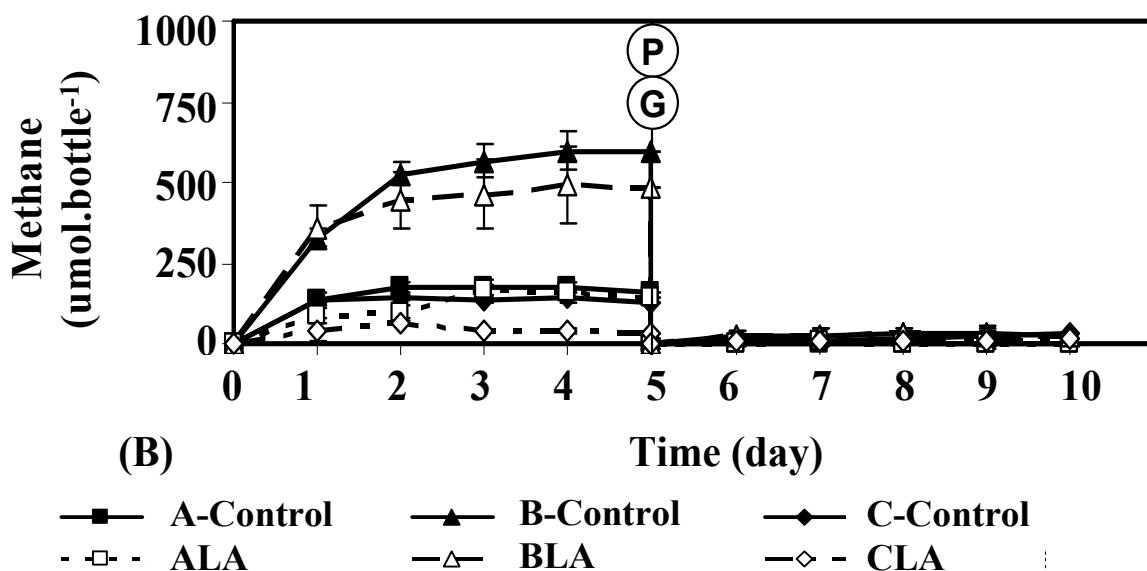


Figure 4.1 Methane production profiles from 5,000  $\text{mg L}^{-1}$  glucose fermentation with and without 2,000  $\text{mg L}^{-1}$  LA in three different cultures (glucose added on Day 0 and Day 5; mean  $\pm$  standard deviations for triplicate samples).

### 4.3.3 H<sub>2</sub> consumption during H<sub>2</sub> production study

Apparent H<sub>2</sub> consumption was observed in controls and LA-treated samples of cultures A, B, and C. This represents the H<sub>2</sub> consumed after the yield reached a peak value during the course of fermentation.

LA decreased the H<sub>2</sub> consumption rates in cultures B and C after the first glucose injection (Table 4.4). In comparison to cultures B and C, culture A did not show H<sub>2</sub> consumption after the first glucose injection in the presence of LA (Table 4.2) because this culture did not reach a peak before the end of the experiment. The H<sub>2</sub> consumption rates in the control versus the LA-treated sample of the same culture were significantly different from each other. Overall, H<sub>2</sub> consumption rates varied greatly as indicated by the large standard deviations recorded in Table 4.4, and generally increased after the second glucose injection in both controls and LA treated cultures.

Table 4.4 Hydrogen consumption rates after glucose injections in cultures A, B, and C. (Mean±standard deviations of triplicate samples are shown).

<u>Type of culture</u>	<u>Experimental condition</u>	<u>H<sub>2</sub> consumption rate (μmol H<sub>2</sub> mg·VSS<sup>-1</sup> day<sup>-1</sup>)</u>	
		<u>1<sup>st</sup> glucose injection</u>	<u>2<sup>nd</sup> glucose injection</u>
Granular brewery plant	AC	0.0±0.0	0.0 ±0.0
	ALA	0.0 ±0.0	1.73±1.45 <sup>a</sup>
Granular ethanol plant	BC	1.95±0.99 <sup>a</sup>	2.96±1.95 <sup>a</sup>
	BLA	1.44±0.89 <sup>a, d</sup>	2.11±0.68 <sup>a</sup>
Flocculent municipal digested	CC	0.83±0.12 <sup>b, d, f</sup>	3.27±1.75 <sup>a</sup>
	CLA	0.31±0.21 <sup>c, e, g</sup>	2.58±1.49 <sup>a</sup>

Note: Values in the same column with different superscripts (a, b, and c) are statistically different (p < 0.05)

Tukey's multiple comparison test (Box *et al.*, 1978) was used to compare H<sub>2</sub> consumption rates in control and LA-treated samples of sludge A, B, and C against each

other. After the first glucose injection, the H<sub>2</sub> consumption rate in the control sample culture A was significantly different from that in the control sample culture B and C. H<sub>2</sub> consumption rates in LA-treated samples were also different from each other after the first glucose injection. After the second glucose injection, the H<sub>2</sub> consumption rate in the culture A, B, and C were not significantly different from each other in both control and LA-treated samples.

#### **4.3.4 Volatile fatty acids production**

The major volatile fatty acids produced were acetate, butyrate, and propionate (Figure 4.2). Control samples accumulated increasing acetate concentrations over ten days of testing (up to approximately 4200, 2670, and 1790 mg L<sup>-1</sup> in cultures A, B, and C, respectively, on Day 10); culture C accumulated almost 42% of the acetate measured in culture A. Acetate accumulated in LA-treated cultures up to approximately 2170, 1880, and 2200 mg L<sup>-1</sup> in cultures A, B, and C, respectively, on Day 10, five days after the second glucose injection.

Control samples from cultures A and B produced propionate quantities up to 1870 and 2240 mg L<sup>-1</sup> on Day 10 (five days after the second glucose injection), while the culture C control did not produce any propionate. Propionate concentration peaked at approximately 400 mg L<sup>-1</sup> on Day 1 (one day after the first glucose injection) and then decreased to 120 mg L<sup>-1</sup> in LA-treated culture A. LA also inhibited the production of propionate in culture B.

Control samples from cultures A and B did not produce butyrate, but culture C accumulated to a maximum of 2960 mg L<sup>-1</sup> (Figure 4.2). LA-treated cultures accumulated butyrate (up to 1540, 2540, and 2230 mg L<sup>-1</sup> in cultures A, B, and C, respectively). Thus,

increased butyrate production was observed in LA-treated samples of cultures A, B, C, and in the control culture C.

The maximum percentages of electron equivalents converted into acetate, butyrate, and propionate, both after the first and second glucose injections, are given in Table 4.5. Note that LA decreased acetate (in cultures B and A), decreased propionate in culture A and prevented its production completely in culture B, while at the same time it increased butyrate production in all cultures except C (where butyrate production was slightly reduced from the levels seen in culture C controls).

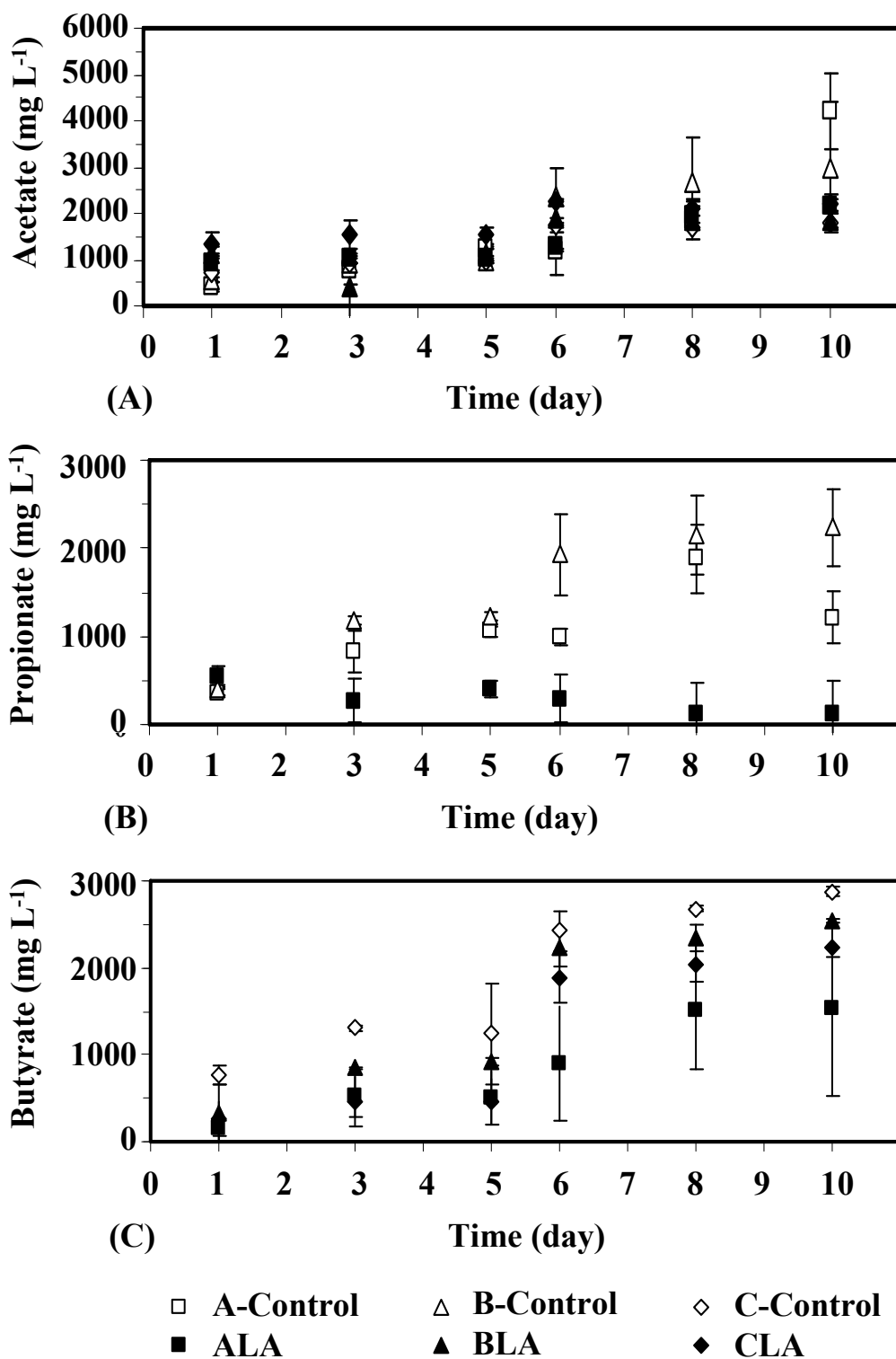


Figure 4.2 Acetate (A), Propionate (B), and Butyrate (C) profiles from 5,000 mg L<sup>-1</sup> glucose fermentation with and without 2,000 mg L<sup>-1</sup> LA for cultures A, B, and C. (glucose added at 0 hr and 120 hr; values shown are Means±standard deviations for triplicate samples).

Table 4.5 Percentage of electron equivalents diverted to by-products on day 5 and 10. (Means±standard deviations of triplicate samples).

By-products	Day	Percent electron equivalents diverted to by-products on day 5 and day 10					
		AC	BC	CC	ALA	BLA	CLA
H <sub>2</sub>	5	13.4±1.0 <sup>a</sup>	3.5±0.0 <sup>b</sup>	23.99±0.67 <sup>c</sup>	9.7±2.6 <sup>d</sup>	5.7±2.8 <sup>d</sup>	6.13±0.91 <sup>d</sup>
	10	19.1±1.4 <sup>a</sup>	13.6±1.7 <sup>b</sup>	20.75±0.75 <sup>a</sup>	18.16±0.74 <sup>d,e</sup>	21.9±2.8 <sup>d</sup>	21.75±0.69 <sup>d,f</sup>
CH <sub>4</sub>	5	3.8±0.4 <sup>a</sup>	14.3±1.4 <sup>b</sup>	3.02±0.36 <sup>c</sup>	3.4±0.4 <sup>d</sup>	11.6±2.8 <sup>e</sup>	0.85±0.15 <sup>f</sup>
	10	1.9±0.2 <sup>a</sup>	9.0±1.0 <sup>b</sup>	1.99±0.31 <sup>a</sup>	2.1±0.2 <sup>d</sup>	7.3±0.4 <sup>e</sup>	0.26±0.16 <sup>f</sup>
HAc	5	25.50±0.8 <sup>a</sup>	19.8±0.6 <sup>b</sup>	19.82±2.01 <sup>b</sup>	20.4±1.3 <sup>d</sup>	22.5±2.4 <sup>d</sup>	30.85±3.84 <sup>e</sup>
	10	42.7±8.4 <sup>a</sup>	26.1±7.1 <sup>b</sup>	18.20±0.82 <sup>b</sup>	22.0±1.5 <sup>d</sup>	18.9±1.6 <sup>d,e</sup>	22.60±1.74 <sup>d,f</sup>
HPro	5	29.9±1.6 <sup>a</sup>	35.0±1.4 <sup>b</sup>	ND	11.5±2.6 <sup>d</sup>	ND	ND
	10	17.3±4.2 <sup>a</sup>	31.8±6.3 <sup>b</sup>	ND	1.7±5.3 <sup>d</sup>	ND	ND
HBut	5	ND	ND	37.87±20.12 <sup>a</sup>	17.4±10.3 <sup>d</sup>	31.5±1.5 <sup>e</sup>	15.75±9.25 <sup>d</sup>
	10	ND	ND	42.47±0.94 <sup>a</sup>	33.0±6.4 <sup>d</sup>	43.8±0.5 <sup>e</sup>	38.39±1.58 <sup>f</sup>
Isopropanol	5	1.5±0.9 <sup>a</sup>	3.7±1.4 <sup>a</sup>	7.77±0.42 <sup>b</sup>	0.9±0.6 <sup>d</sup>	3.3±1.6 <sup>e</sup>	7.77±2.81 <sup>f</sup>
	10	3.8±0.2 <sup>a</sup>	10.9±4.2 <sup>b</sup>	12.54±0.00 <sup>b</sup>	3.4±3.3 <sup>d</sup>	10.8±4.6 <sup>e</sup>	12.54±0.00 <sup>e</sup>
Propanol	5	22.9±6.8 <sup>a</sup>	10.8±4.3 <sup>b</sup>	19.38±2.44 <sup>a</sup>	16.3±1.1 <sup>d</sup>	6.7±3.2 <sup>e</sup>	9.48±2.54 <sup>e</sup>
	10	23.4±9.8 <sup>a</sup>	12.97±0.7 <sup>b</sup>	9.34±0.00 <sup>c</sup>	12.4±0.8 <sup>d</sup>	5.1±1.2 <sup>e</sup>	9.23±0.00 <sup>f</sup>
Isobutanol	5	ND	4.2±0.8 <sup>a</sup>	ND	ND	2.1±0.1 <sup>d</sup>	ND
	10	ND	3.1±1.4 <sup>a</sup>	ND	ND	3.2±0.2 <sup>d</sup>	ND
Ethanol	5	0.03±0.08 <sup>a</sup>	ND	ND	0.96±0.28 <sup>d</sup>	1.8±0.6 <sup>d</sup>	ND
	10	0.8±0.5 <sup>a</sup>	ND	ND	ND	ND	ND

Note:

1. ND = Not detected.
2. AC, BC, and CC = control cultures A, B, and C, respectively. ALA, BLA, and CLA = LA treated samples from cultures A, B, and C, respectively.
3. Tukey's comparison for AC, BC, and CC or ALA, BLA, and CLA in the same row.
4. For AC, BC, and CC, values with the different superscripts (a, b and c) are statistically different ( $p < 0.05$ ). The same applies to ALA, BLA, and CLA (superscripts include d, e and f).
5. The percentage of electron equivalents consumed into biomass growth was assumed to be 10%.

### 4.3.5 Alcohol production

Alcohols were produced by all cultures, likely because of the low pH and a slight shift from acidogenesis to solventogenesis upon decreasing the pH. The major alcohols detected included ethanol, isopropanol, propanol, and isobutanol. Figure 4.3 shows alcohol profiles under the different incubation conditions. In general, production increased for isopropanol and propanol over time. No particular trend was observed for ethanol production. Alcohols produced during glucose degradation were not degraded in



the control or LA-treated samples over 10 days of sampling, except for the small reduction in ethanol concentration seen in LA-treated samples of culture B (Figure 4.3).

Isopropanol was produced in all of the experimental conditions. After the first glucose injection, a small amount of isopropanol ( $< 200 \text{ mg L}^{-1}$ ) was produced in the control and LA-treated samples of cultures A, B, and C. After the second glucose injection, LA-treated samples of culture C accumulated an increasing concentration of isopropanol (up to  $1060 \text{ mg L}^{-1}$ ), whereas control samples from the same culture produced a maximum of  $560 \text{ mg L}^{-1}$ . Culture B treated with LA produced comparable concentrations of isopropanol to those produced in the control samples of culture C. Control and LA-treated samples of culture A produced less isopropanol than the other cultures; a maximum of 140 and  $170 \text{ mg L}^{-1}$  were produced in control and LA-treated samples of culture A, respectively, on Day 10 (five days after the second glucose injection).

Small quantities of ethanol were produced in LA-treated samples of culture A and B. No ethanol was detected in culture C. A maximum of  $50 \text{ mg L}^{-1}$  ethanol was measured in LA-treated samples of culture B on Day 5 (five days after the first glucose injection). Control samples of culture A produced a maximum level of ethanol ( $40 \text{ mg L}^{-1}$ ) on Day 10 (five days after the second glucose injection), whereas, LA-treated samples of the same culture produced a maximum of  $20 \text{ mg L}^{-1}$  of ethanol four days after the second glucose injection (Day 9).

Increasing quantities of propanol were produced in cultures A, B and C over the duration of the study. The maximum concentration ( $1040 \text{ mg L}^{-1}$ ) of propanol was produced in the control samples of culture A. Five days after the second glucose injection (Day 10), the LA-treated samples of culture A produced a maximum of  $550 \text{ mg L}^{-1}$  of

propanol. Culture B samples treated with LA produced less propanol in comparison to samples from other cultures; for example, a maximum level of 230 mg L<sup>-1</sup> of ethanol was present on Day 10 (five days after the second glucose injection). One day after the second glucose injection (Day 6), LA-treated samples of culture C produced a maximum amount of 450 mg L<sup>-1</sup>.

Isobutanol was detected in control and LA-treated samples of culture B. A maximum concentration of 170 mg L<sup>-1</sup> was measured in LA-treated samples of culture B on Day 10 (five days after the second glucose injection).

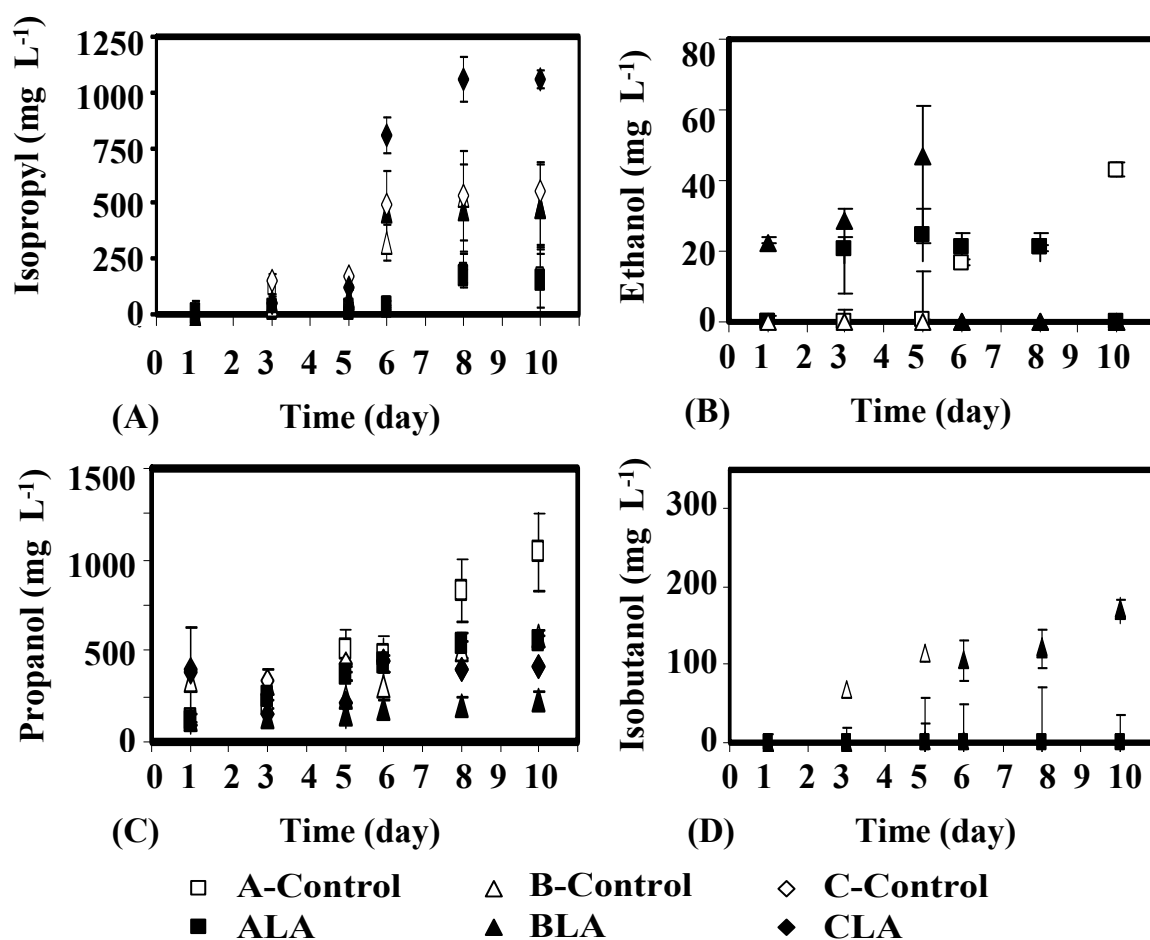


Figure 4.3 Alcohol production: Isopropanol (A), Ethanol (B), Propanol (C), and Isobutanol (D) profiles from 5,000 mg L<sup>-1</sup> glucose fermentation with and without 2,000 mg L<sup>-1</sup> LA for cultures A, B, and C. (Glucose added at 0 hr and 120 hr; values shown are mean  $\pm$  standard deviations for triplicate samples).

### 4.3.6 Glucose consumption

Glucose degradation profiles in control and LA-treated cultures A, B, and C are shown in Figure 4.4. Generally, LA decreased the rate of glucose degradation in all cultures. After 12 hrs, 57%, 44%, and 52% of the glucose was consumed in the control samples of sludge A, B, and C respectively, whereas, 34%, 21%, and 42% was consumed in LA-treated samples of the three cultures. During the first 12 hours of incubation, glucose consumption in LA-treated cultures was 60%, 48%, and 81% of that consumed in control samples of cultures A, B, and C, respectively (Figure 4.4).

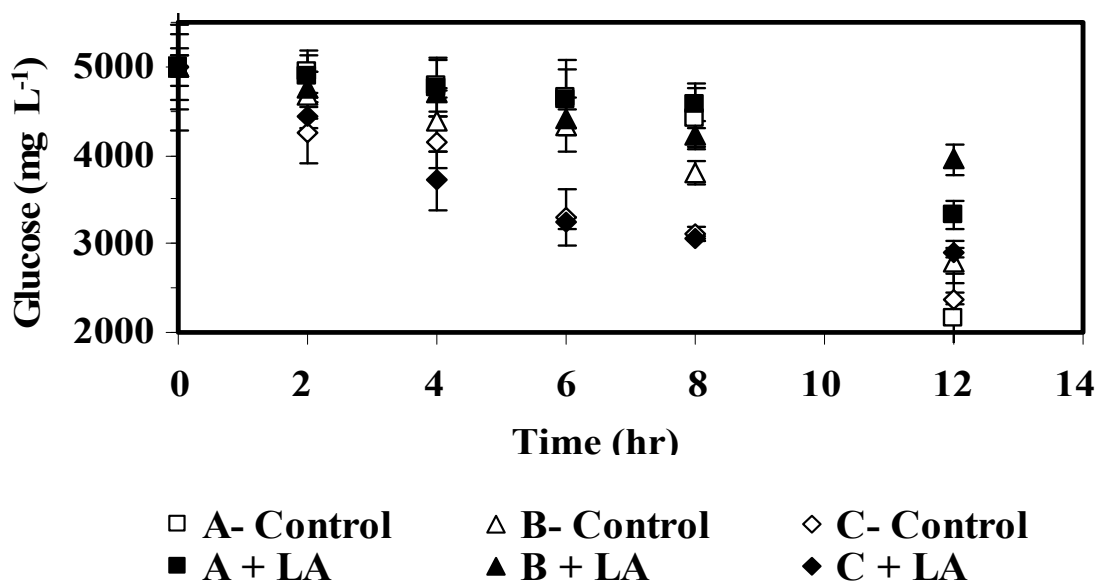


Figure 4.4 Glucose degradation profiles for control and LA-treated cultures A, B, and C receiving 5,000 mg L<sup>-1</sup> glucose plus 2,000 mg L<sup>-1</sup> LA. (Values shown are means±standard deviations for triplicate samples).

The initial degradation rates for the different conditions examined were used to assess the impact of LA on the glucose consumption by acidogens (Table 4.6). The initial degradation rates in the control samples of cultures A, B, and C decreased by 23%, 69%, and 37%, respectively when LA was present. The mean values of the glucose initial degradation rates were compared using Tukey's paired multiple comparison procedure at

95% confidence level (Box *et al.*, 1978). Differences as well similarities for initial glucose degradation rates were observed for the control and LA-treated cultures (Table 4.6). The data demonstrated that the addition of LA reduced the initial glucose degradation rates to differing degrees in the three cultures.

Table 4.6 Glucose initial degradation rate in controls and LA-treated samples of cultures A, B, and C.

<b>Culture</b>	<b>Glucose initial degradation rate (<math>\mu\text{g glucose mg}^{-1} \text{ VSS min}^{-1}</math>)</b>	
	<b>Control</b>	<b>LA</b>
A	1.85±0.21 <sup>a</sup>	1.42±0.22 <sup>a</sup>
B	1.73±0.43 <sup>a</sup>	0.53±0.17 <sup>b</sup>
C	4.01±0.47 <sup>b</sup>	2.53±1.00 <sup>c</sup>

Tukey's comparison for AC, BC, and CC or ALA, BLA, and CLA in the same column. Means with different superscripts (a, b, and c) are statistically different ( $p < 0.05$ ).

#### 4.3.7 Modeling the kinetics of H<sub>2</sub> production

A modified Gompertz equation [Eq. (4.1)] was used to describe the progression of H<sub>2</sub> production and to fit the cumulative H<sub>2</sub> production curves for each culture under the different experimental conditions. The modified Gompertz model can provide constants with biological meanings ( the maximum H<sub>2</sub> production rate, H<sub>2</sub> production potential, and duration of the lag phase. This information can be used to enable a better understanding of the process (Wang *et al.*, 2009). The formula of the modified Gompertz model is:

$$H = P \exp \left\{ - \exp \left[ \frac{R_m \cdot e}{P} (\lambda - t) + 1 \right] \right\} \quad (4.1)$$

where  $H$  is the cumulative  $H_2$  production (mL),  $\lambda$  the lag-phase time (hr),  $P$  is the  $H_2$  production potential (mL),  $R_m$  is the maximum  $H_2$  production rate (mL h<sup>-1</sup>),  $t$  is the incubation time (h),  $e$  the exp (1) = 2.718. The  $R^2$  value for all modeled data sets ranged from 0.9408 to 0.9831. Parameters used in fitting the experimental data to Eq. 4.1 are given in Table 4.7.

Table 4.7 Parameters used in the Gompertz model for the three cultures (data shown for the 2<sup>nd</sup> glucose injection).

<b>Sludge</b>	<b>LA (mg L<sup>-1</sup>)</b>	<b>P (mL)</b>	<b>R<sub>m</sub> (mL h<sup>-1</sup>)</b>	<b><math>\lambda</math> (h)</b>	<b>R<sup>2</sup></b>
<b>A</b>	0	19.42	0.81	48	0.9831
<b>A</b>	2,000	66.15	2.89	24	0.9649
<b>B</b>	0	61.84	2.58	0	0.9408
<b>B</b>	2,000	97.21	4.05	0	0.9732
<b>C</b>	0	98.38	4.10	0	0.9522
<b>C</b>	2,000	100.5	4.19	0	0.9877

The Gompertz model described the progression of  $H_2$  production in controls and LA-treated samples of cultures A, B, and C (Figure 4.5) with an  $R^2$  value between 0.94 and 0.98 (Table 4.7). Note that Figure 4.5 indicates that a modified Gumpertz model equation is insensitive to  $H_2$  consumption and does not account for any loss in the modeled parameter after the observed peak.

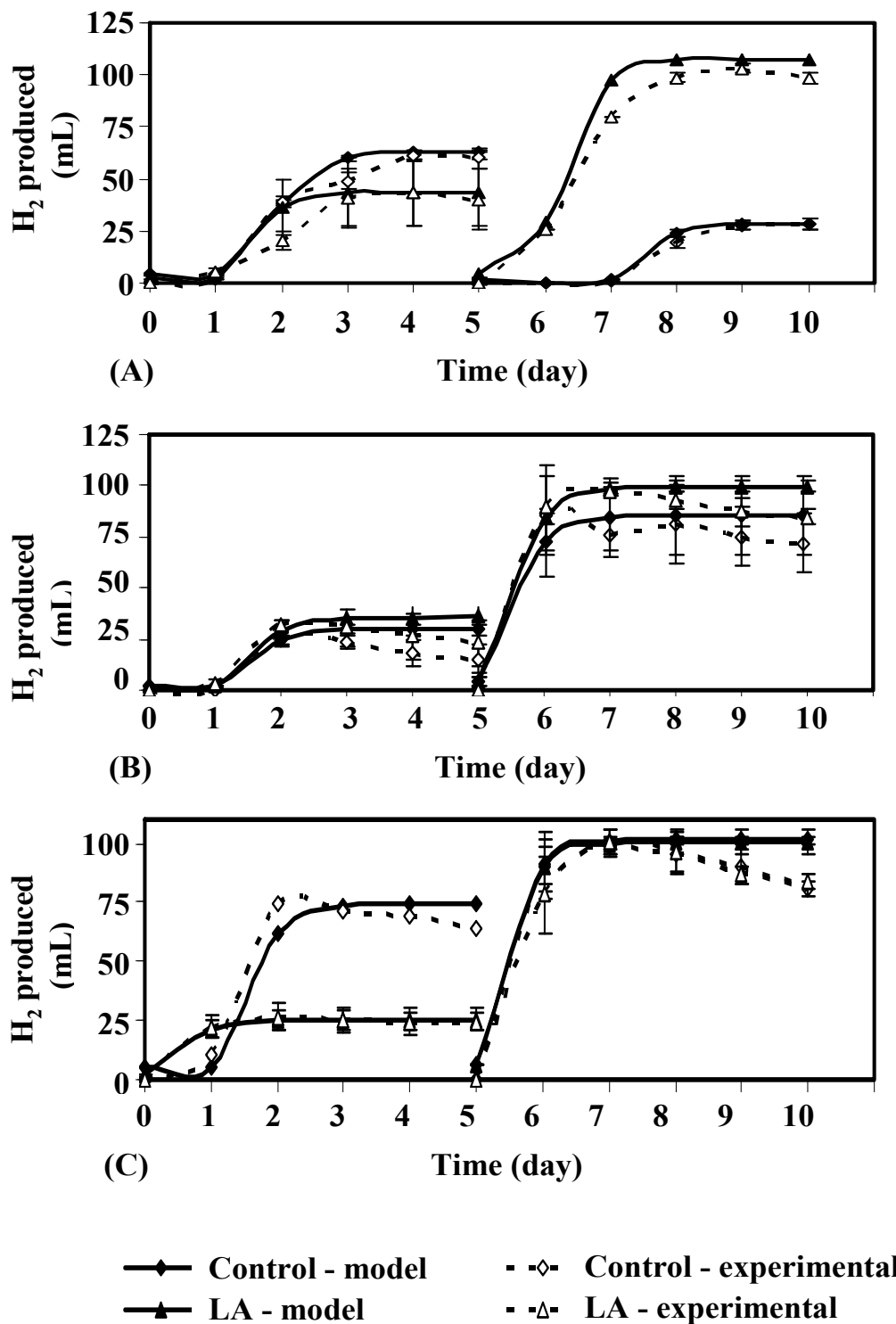


Figure 4.5 Comparison of experimental hydrogen production to predicted hydrogen production from the Gompertz model for (A) culture A, (B) culture B, and (C) culture C. (Values shown are mean $\pm$ standard deviations of triplicate samples).

#### 4.3.8 Electron mass balance

The electron mass balance for controls and 2,000 mg L<sup>-1</sup> LA-treated samples of cultures A, B and C are presented in Figure 4.6. The procedure used in the electron mass balance and sample calculation are given in Appendix D. Electron percentage recovery was approximately 100% on five days after the first (Day 5) and second (Day 10) glucose injections. Recovery of greater than 100% of the electrons might have been due to possible biomass decay into soluble by-products which were utilized by surviving microorganisms at the end of the fermentation. The electron mass balance data show that an increased percentage of electron equivalents were directed towards H<sub>2</sub> in cultures treated with LA compared to controls, except in culture C after the first glucose injection when the opposite was observed. In this case, electron equivalents channelized to H<sub>2</sub> after the first glucose injection accounted for more than 23% of the total electron equivalents available in glucose. After the second glucose injection, all LA-treated cultures channelized more electron equivalents to H<sub>2</sub> in comparison to their control cultures.

Five days after the first and second glucose injections (Days 5 and 10, respectively), almost 70% of electron equivalents were accounted for in VFAs produced in control and LA-treated samples of cultures A, B, and C. The control cultures A, B, and C directed significantly more electron equivalents towards alcohol production than LA-treated cultures (Table 4.5, Figure 4.6D, and Figure 4.7); a maximum of 23% of the electron equivalents in glucose were synthesized in alcohols in control cultures, whereas in LA-treated cultures it was only 18%.

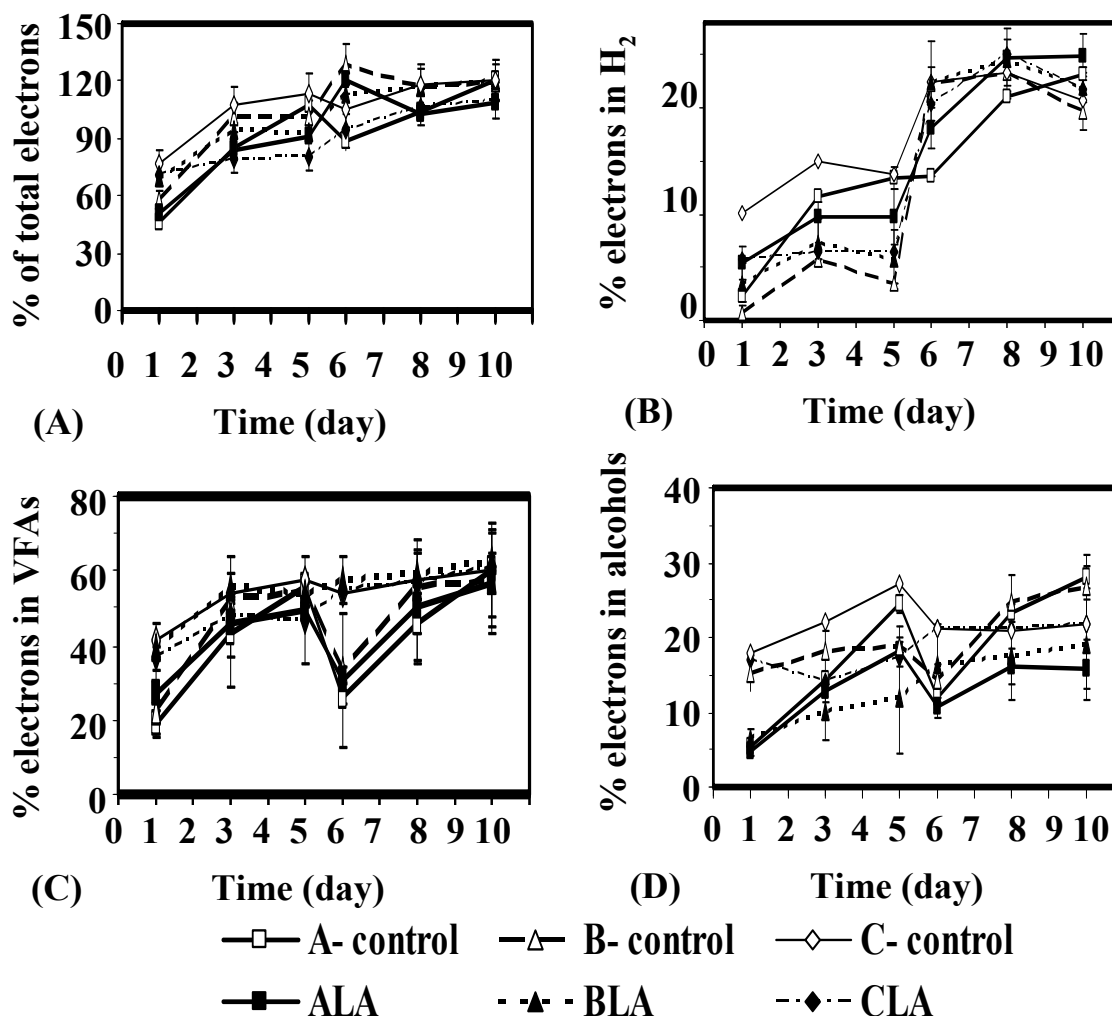
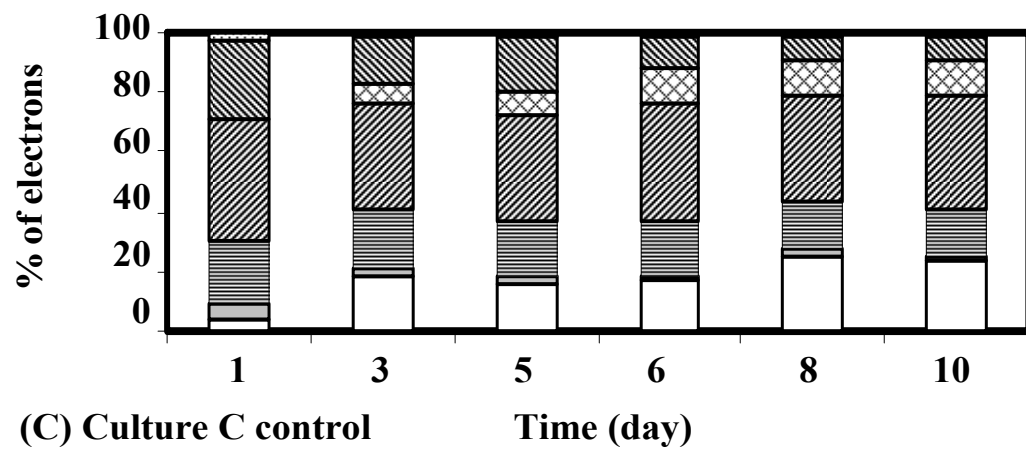
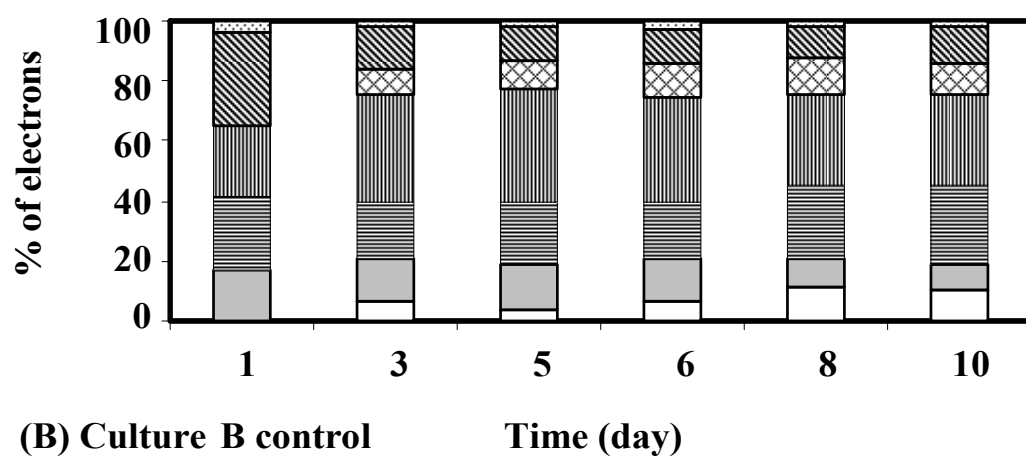
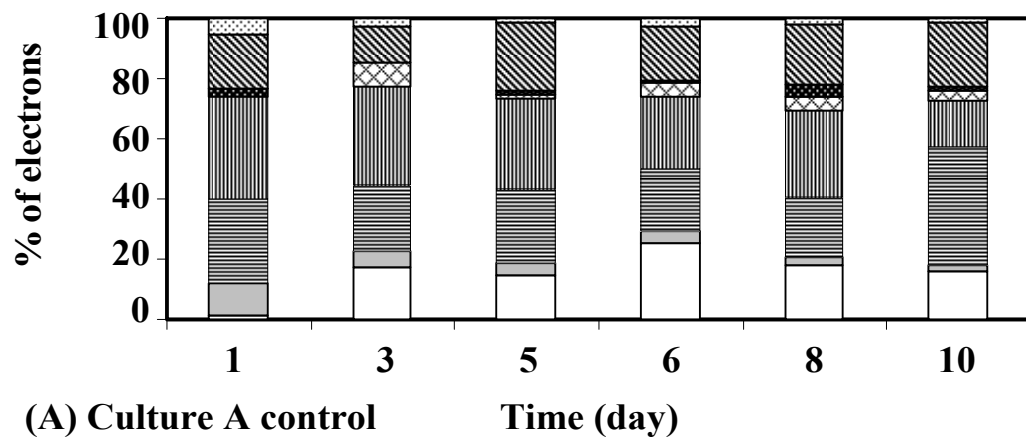


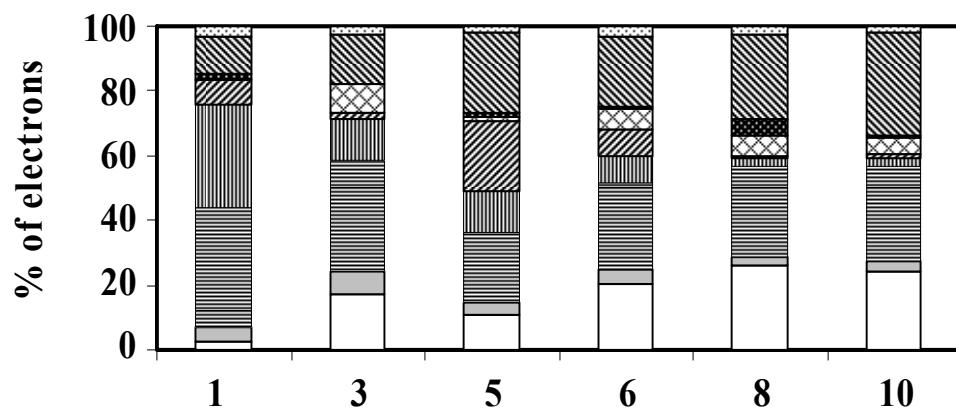
Figure 4.6 Electron mass balance for controls and LA-treated cultures A, B, and C. All cultures treated with 5,000 mg L<sup>-1</sup> glucose. (Values shown are means and standard deviations for triplicate samples are shown).

The distribution of the electrons in the various species of by-products on specific days is shown in Figure 4.7. Propionate and acetate held most of the electron equivalents in culture A and B control samples, whereas butyrate and acetate were dominant in culture C. LA affected the distribution of the electron equivalents and directed them towards  $H_2$  and butyrate instead of propionate in culture B.



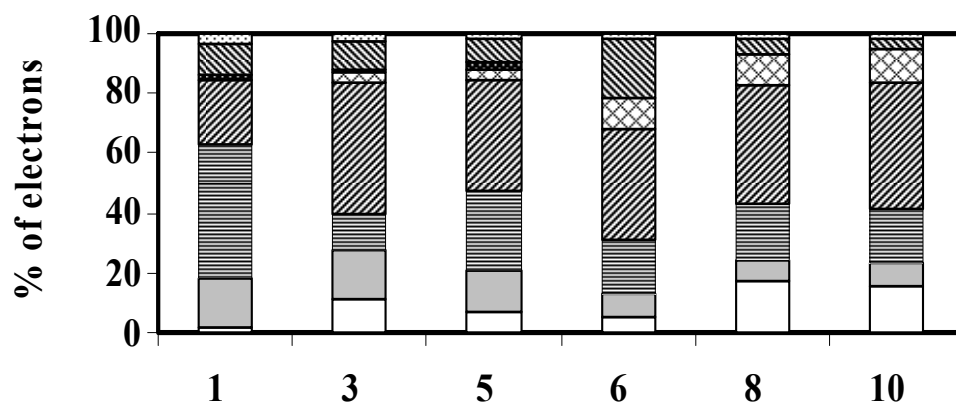


□ H2	■ CH4	■ Acetate
■ Propionate	■ Butyrate	■ Isoprpyl
■ Ethanol	■ Propyl	■ Biomass



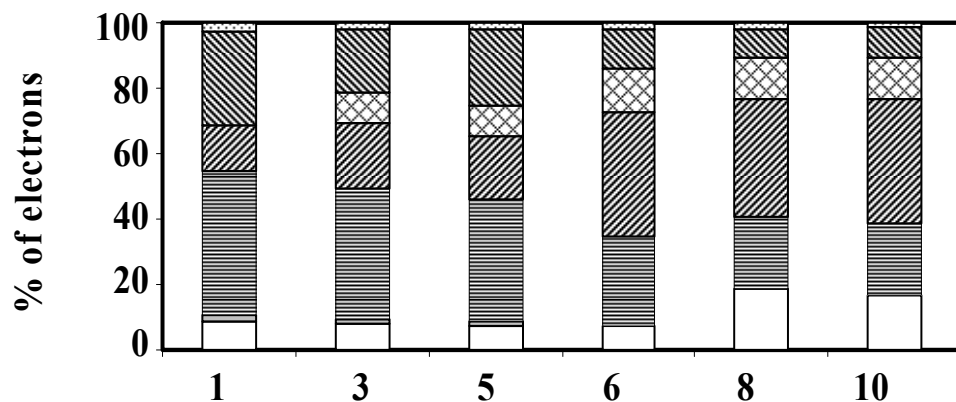
(D) Culture A + LA

Time (day)



(E) Culture B + LA

Time (day)



(F) Culture C + LA

Time (day)



Figure 4.7 Daily distributions of electron equivalents in the by-products for control and LA-treated cultures A, B and C fed 5,000 mg L<sup>-1</sup> glucose plus 2,000 mg L<sup>-1</sup> LA.

#### 4.3.9 Flux balance analysis

Mass balance analyses of the metabolic fluxes on Days 5 and 10 were conducted according to the metabolic reactions network model shown in Figure 3.3 using MetaFluxNet software, Version 1.8.6.2. The dashed line in Figure 3.3 represents the cell wall and distinguishes the intercellular and extracellular metabolites. The stoichiometries of the reactions (R1 to R30) are given in Table 3.9. The input included the external substrate (glucose) and the measured by-products (gases, VFAs, and alcohols). The output of the metabolic flux analysis in cultures A, B, and C on Day 5 is given in Figure 4.8(A) and (B) for the control and LA treated samples, respectively. The same analysis was also conducted on Day 10; the results are shown in Figure 4.9(A) and (B) for the controls and LA treated cultures, respectively.

The flux balance analysis explains the various metabolic routes where  $H_2$  is consumed. Five days after the first glucose injection (Day 5; Figure 4.8), it is very clear from reactions R12 and R13 that the quantities of  $H_2$  produced were higher than the quantities measured in the batch reactors' headspace. The differences can be explained by reactions R17, R21, and R29. The acetogenic consumption of  $H_2$  (R17) is generally higher than the solventogenic consumption (R21) because of the difference in the stoichiometry of  $H_2$  in these two reactions. Methanogenesis also consumed a significant amount of  $H_2$  both in control and LA-treated samples of culture B.

Five days after the second glucose injection (i.e., Day 10 of the experiment; Figure 4.9), it is clear from reactions R12 and R13 that the quantities of  $H_2$  consumed were

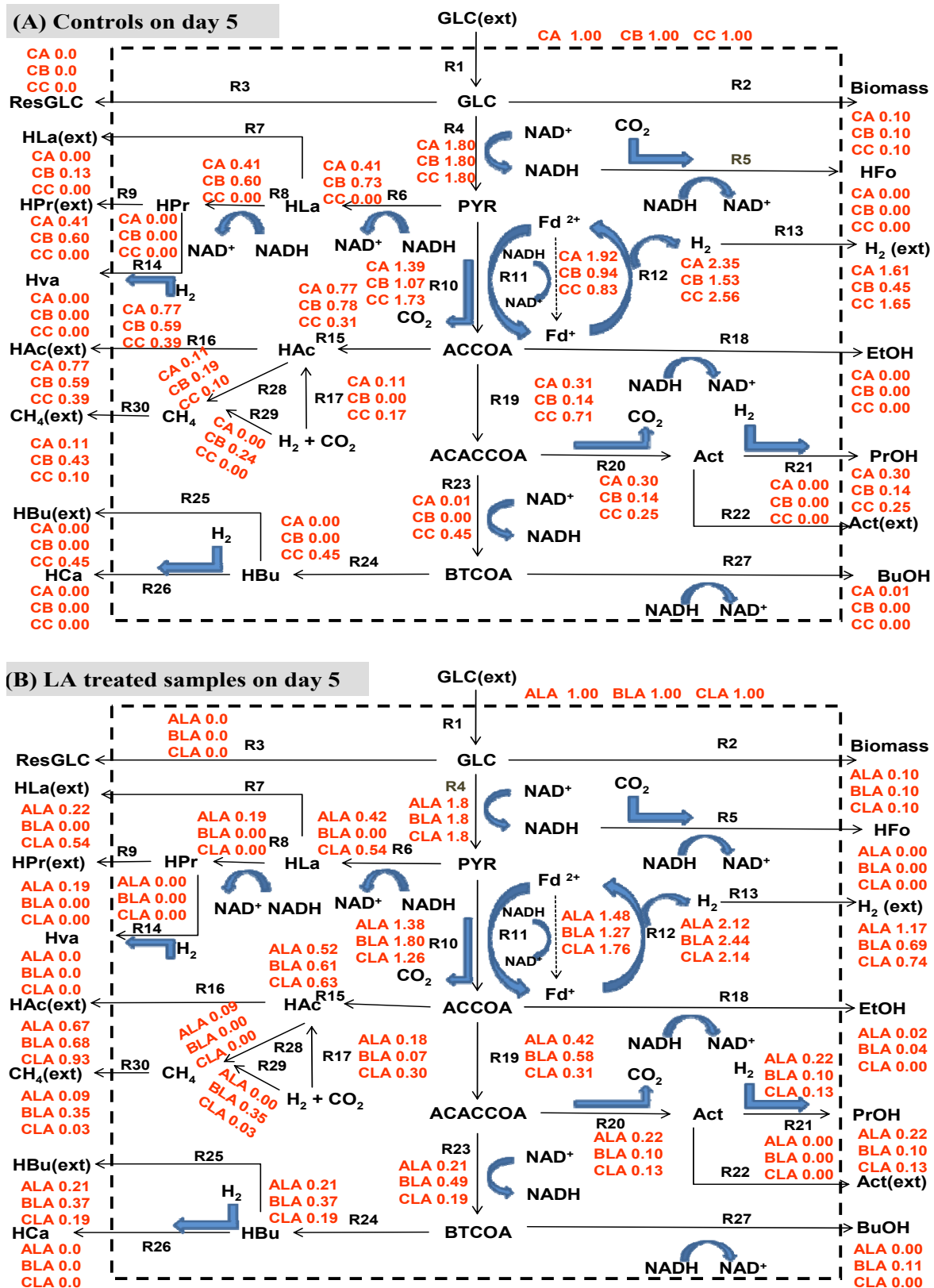
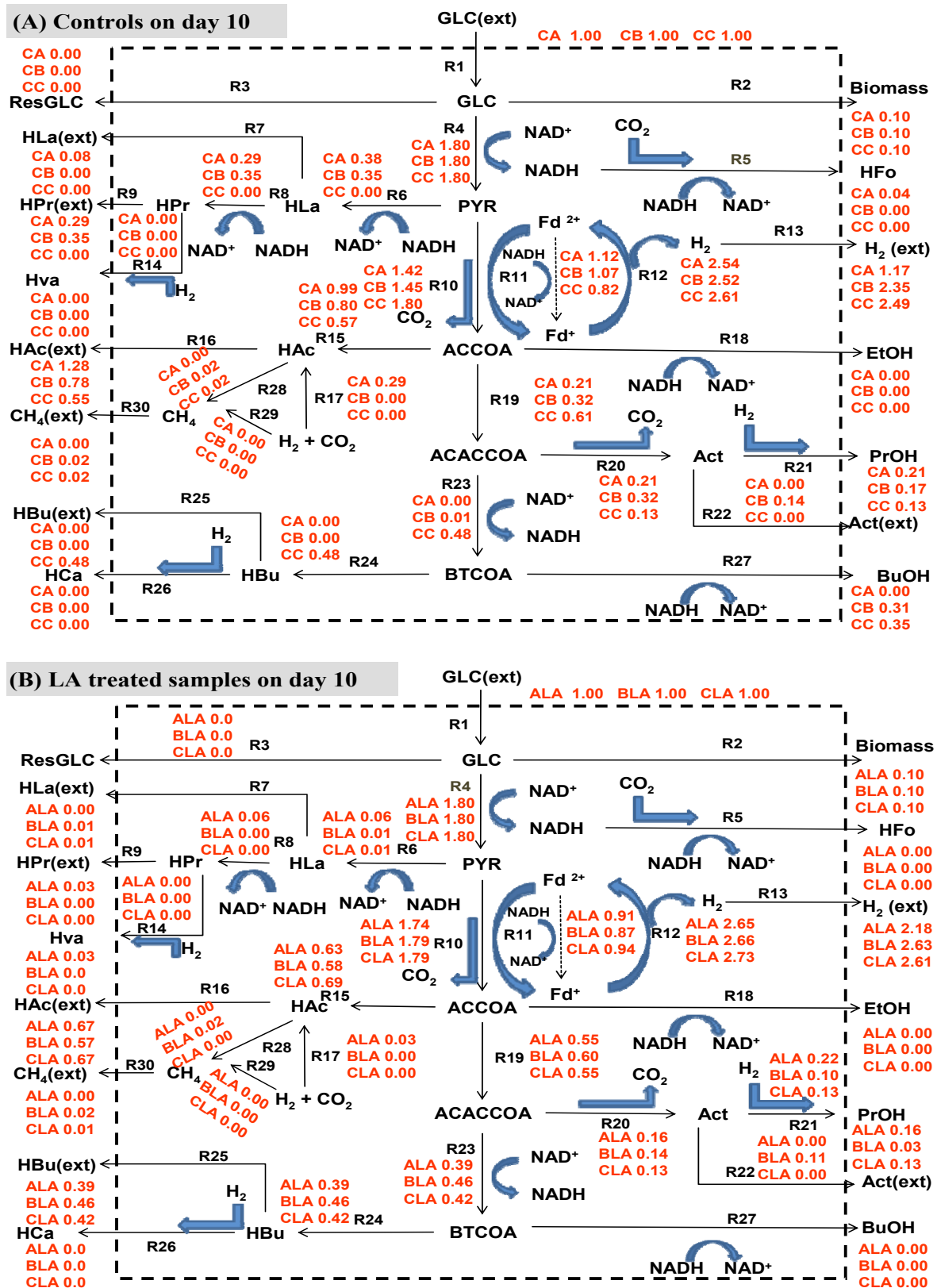


Figure 4.8 Flux balance analysis for culture A, B, and C on day 5 in (A) control and (B) LA-treated samples.



smaller in comparison to five days after the first glucose injection (Figure 4.8). Except for the control culture A, the acetogenic consumption of  $H_2$  (R17) was eliminated, and the solventogenic consumption (R21) accounted for most of the  $H_2$  consumed. Methanogenesis was completely inhibited after the second glucose injection.

#### **4.3.10 Principal components analysis of the by-products**

Principal components analysis (PCA) reduced the number of variables needed to compare different samples and detected similarities or differences among the various controls and LA-treated cultures A, B, and C (Statistica ® software package; version 7.0). PCA was conducted using three data sets: the first comprised of  $H_2$  yield only (Figure 4.10); the second included all by-products (Figure 4.11); and the third (Figure 4.13) used the data set from the metabolic flux analysis output (Figure 4.8 and Figure 4.9) to detect similarities or differences among the three cultures in terms of the intercellular metabolites.

The first PCA (Figure 4.10) included six cases (samples AC, BC, CC, ALA, BLA, and CLA) and one variable ( $H_2$  yield); the first two components accounted for 89 % of the variation in the data set. The results indicate that LA changed the response of the three cultures in terms of  $H_2$  yield in the same way. Culture A, B, and C treated with 2,000 mg  $L^{-1}$  LA clustered together (enclosed and shaded in Figure 4.10) and were very distinct from their controls. Notice the differences among the control samples of the three cultures as indicated by the great distances between any two of them on PC1 and PC2 projections.

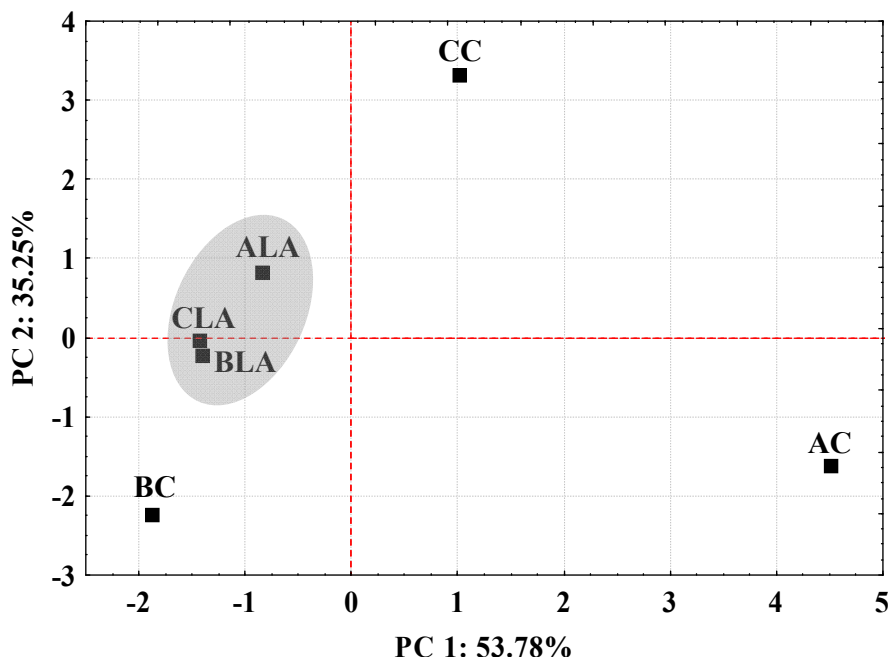
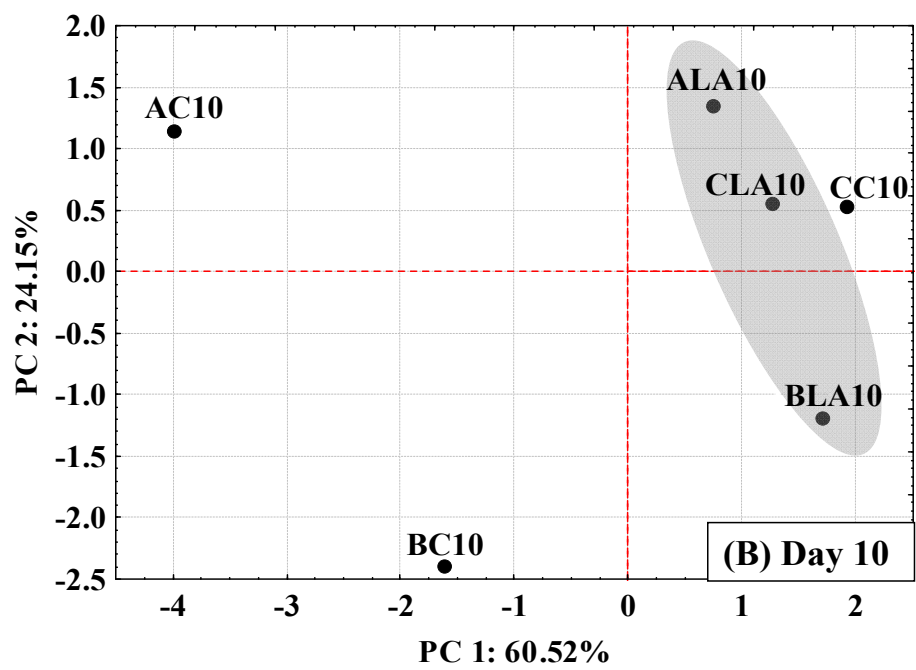
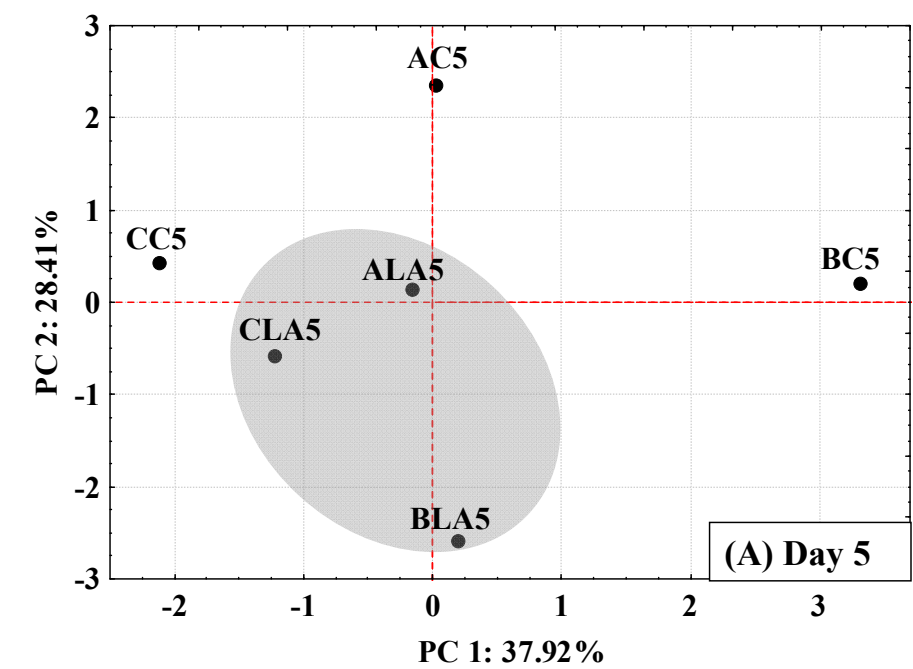


Figure 4.10 Principal component analysis for H<sub>2</sub> yield data set for controls and 2,000 mg L<sup>-1</sup> LA-treated cultures A, B, and C. All cultures received 5,000 mg L<sup>-1</sup> glucose.

The second PCA (Figure 4.11) included 9 variables (H<sub>2</sub>, CH<sub>4</sub>, acetate, propionate, butyrate, isopropanol, ethanol, propoanol, and isobutanol) and 6 cases (AC, BC, CC, ALA, BLA, CLA), and was conducted using by-product data sets from Day 5, Day 10, and Day 5 and Day 10 combined. The first two principal components accounted for 66%, 85%, and 64 % of the variation in the three data sets (Day 5, Day 10, and Day 5 and 10 combined). The second PCA supports the first PCA in that LA altered the behavior of the three cultures in a similar manner. LA-treated cultures were more similar to each other than they were to their respective controls. Notice how the LA-treated cultures are clustered together in each of the analyses (Figure 4.11 - A, B, and C). The loadings maps of variables (Figure 4.12) show the inverse relationship between CH<sub>4</sub> and propionate production and H<sub>2</sub> yield. Moreover, Figure 4.12 also indicates the direct relationship of H<sub>2</sub> yield with acetate and butyrate production.





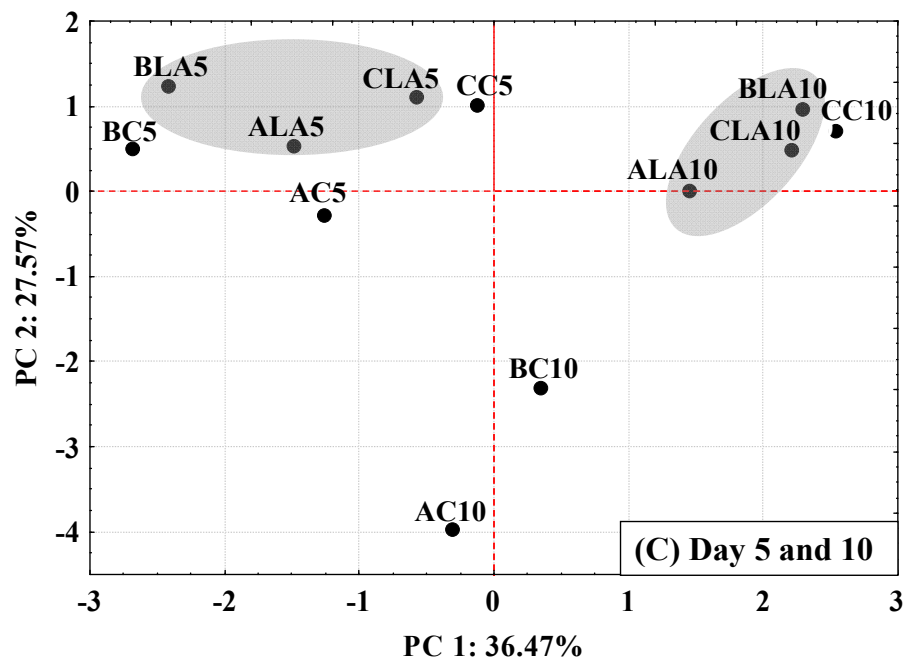
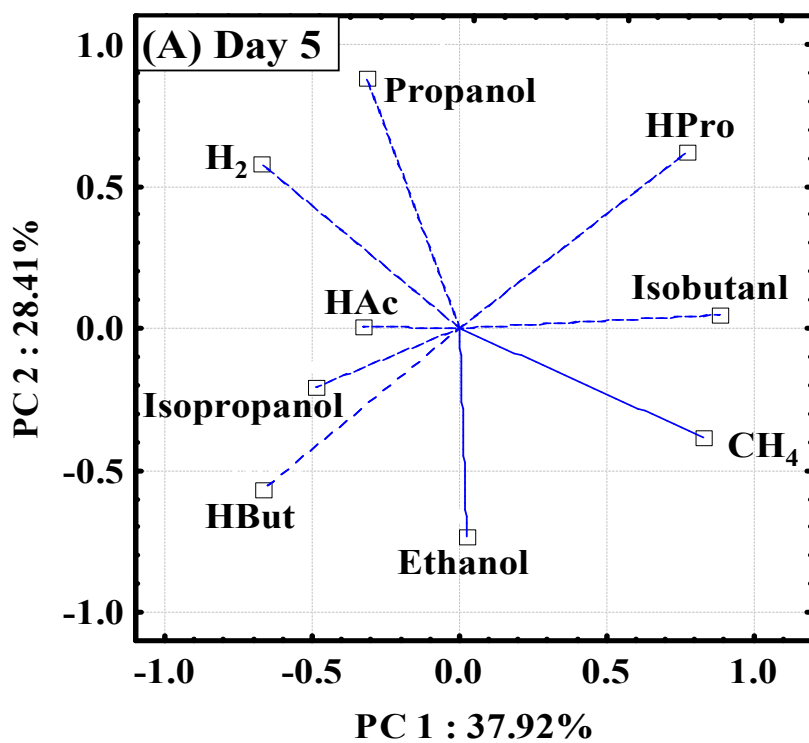


Figure 4.11 Score maps of principal component analyses for all by-products data set on day (A) 5, (B) 10, and (C) 5 and 10 for control and 2,000 mg L<sup>-1</sup> LA-treated samples of cultures A, B, and C (All cultures received 5,000 mg L<sup>-1</sup> glucose).



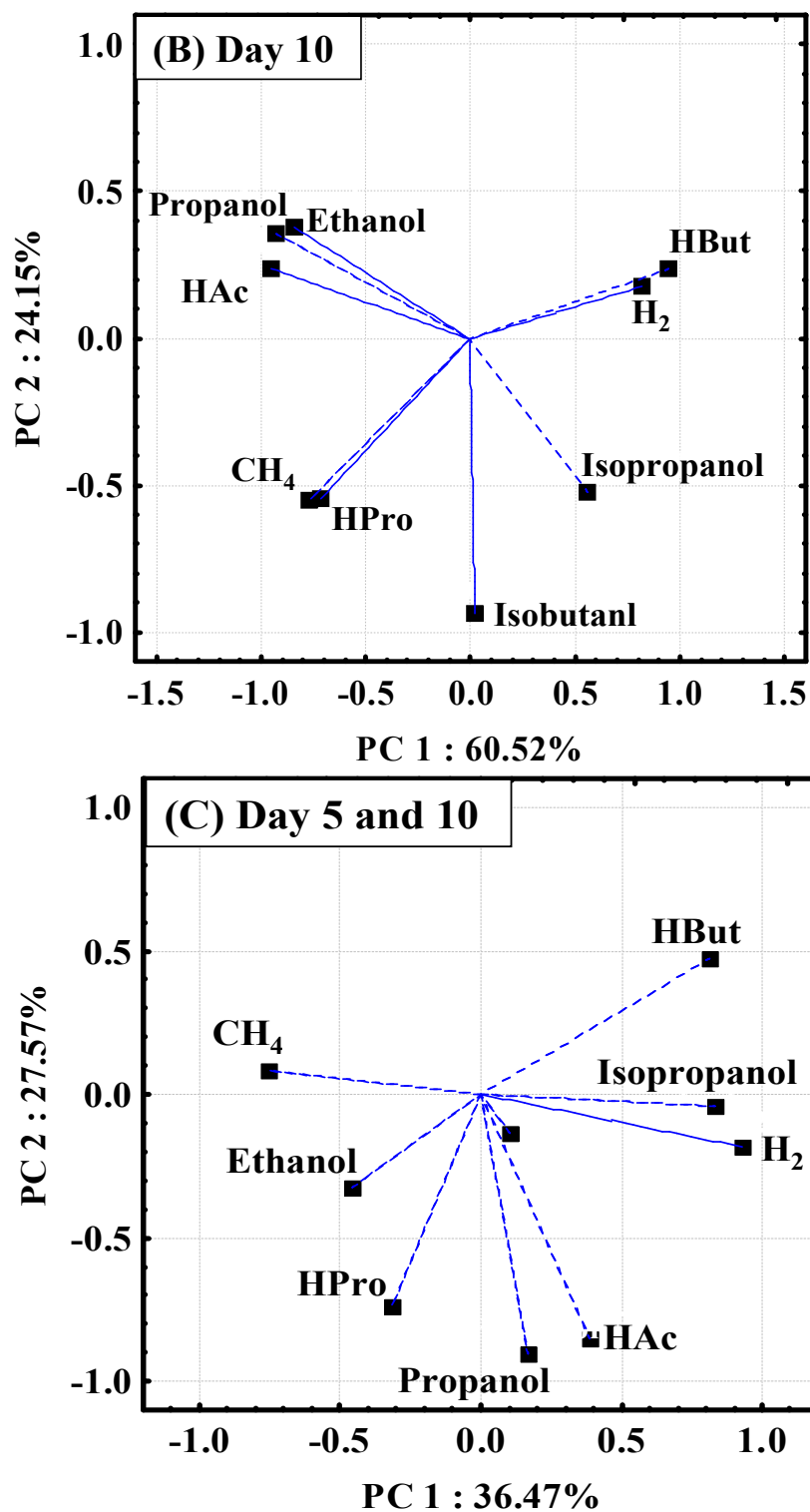
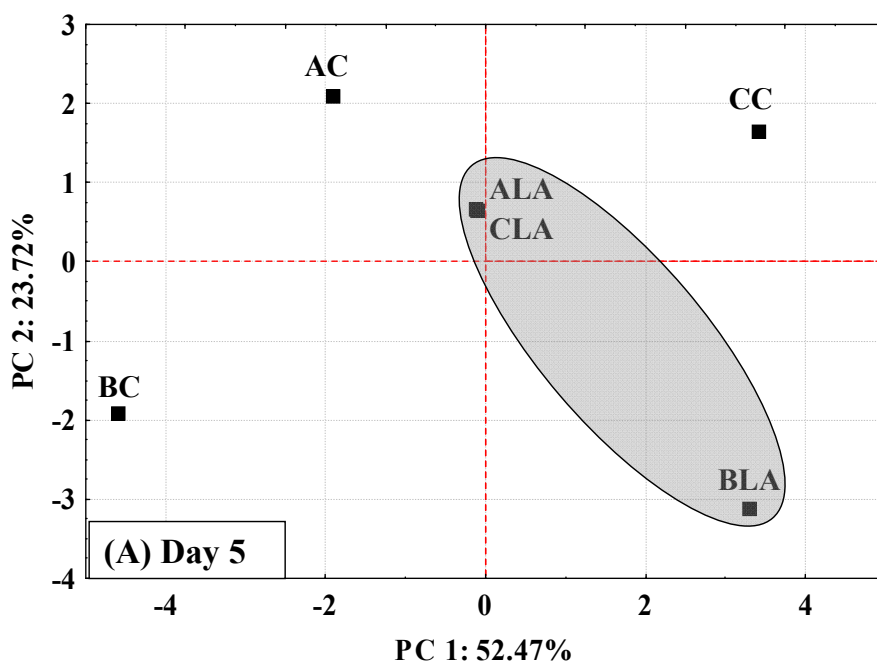


Figure 4.12 Loadings maps of principal component analyses for the data sets of all by-products on days (A) 5, (B) 10, and (C) 5 and 10 combined for control and 2,000 mg L<sup>-1</sup> LA-treated samples of cultures A, B, and C (All cultures received 5,000 mg L<sup>-1</sup> glucose).

The third PCA of the data set from the metabolic flux analysis output on days 5 and 10 included 6 cases (AC, BC, CC, ALA, BLA, CLA) and the variables were lactate, propionate, pyruvate, ferredoxin, hydrogen, acetate, ethanol, acetoacetylCoA, acetone, propanol, butyrylCoA, butyrate, butanol, methane (i.e., the output of the reactions: R6, R7, R8, R9, R10, R11, R12, R15, R17, R18, R19, R20, R21, R23, R24, R27, R28, and R29); the first two components accounted for 76% and 90 % of the variance in the Day 5 and Day 10 data sets, respectively. The results indicated that after the treatment with LA, the pool and distribution of intercellular metabolites in cultures A, B, and C were similar (Figure 4.13). The degree of similarity increased after the second glucose injection as BLA moved closer to ALA and CLA, whereas the differences among the control cultures remained.



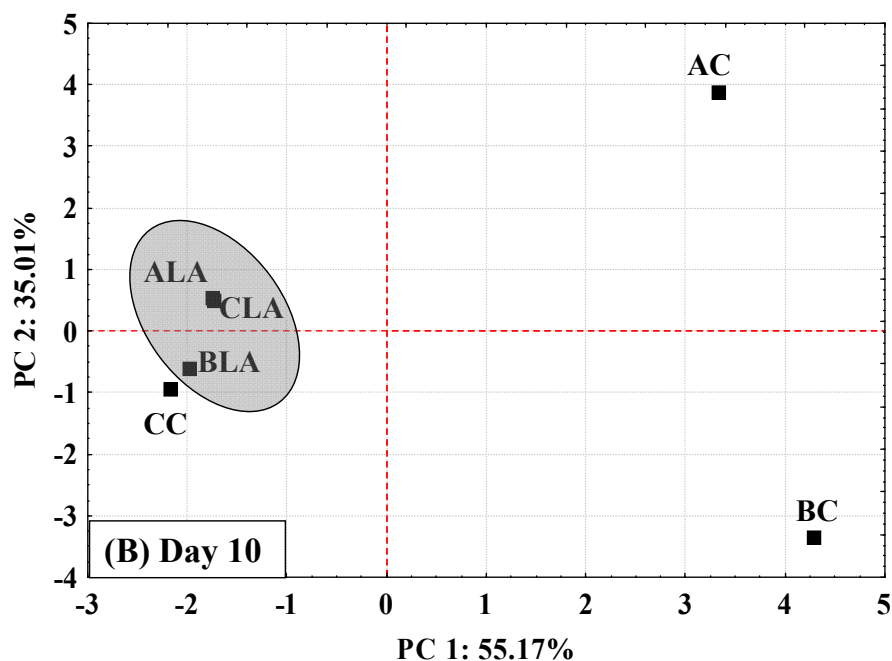


Figure 4.13 Score maps of principal component analysis for the output of the metabolic flux analysis on day (A) 5 and (B) 10, for control and 2,000 mg L<sup>-1</sup> LA-treated samples of cultures A, B, and C (All cultures received 5,000 mg L<sup>-1</sup> glucose).

#### 4.4 DISCUSSION

Mixed anaerobic cultures with and without pretreatment produced H<sub>2</sub> under various stress conditions such as low pH, heat pre-treatment and chemical inhibitors of methanogens. However, H<sub>2</sub> yields from different cultures under the same stress conditions varied greatly (0.9 to 2.0 mol H<sub>2</sub>·mol<sup>-1</sup> glucose, Table 4.8). Valdez-Vazquez and Poggi-Varaldo (2008) attributed differences in H<sub>2</sub> yield to the different populations of microorganisms in each inoculum. The present investigation demonstrated that treatment with LA, a non-toxic biodegradable natural organic polymer, could overcome differences in the microbial communities found in a variety of mixed cultures and could bring their H<sub>2</sub> yields to comparable levels. The H<sub>2</sub> yield from LA-treated methanogenic

cultures (A and B) were higher than the yield reported by Wang *et al.* (2007) in heat-treated methanogenic culture.

Table 4.8 Hydrogen yield from glucose in batch studies.

Inoculum	Conditioning method	H <sub>2</sub> yield*	Reference
Anaerobic sludge	2,000 mg L <sup>-1</sup> LA	3.11	This work
Anaerobic sludge	2,000 mg L <sup>-1</sup> LA	2.4	Ray <i>et al.</i> , 2008
Anaerobic sludge	2,000 mg L <sup>-1</sup> LA	1.7	Chowdhury <i>et al.</i> , 2007
Anaerobic sludge	Heat treatment	1.75	Zheng and Yu, 2005
Anaerobic sludge	Acid treatment	0.8-1.0	Cheong and Hansen, 2006
Aerobic and anaerobic sludges, soil and lake sediment	Acid and heat treatment	1.4	Kawagoshi <i>et al.</i> , 2005
Anaerobic sludge	Heat treatment	2.0	Park <i>et al.</i> , 2005
Anaerobic sludge	Heat treatment	1.17	Salerno <i>et al.</i> , 2006
Anaerobic sludge	Heat treatment	0.97	Oh <i>et al.</i> , 2003
Soil microflora	Heat treatment	0.92	Logan <i>et al.</i> , 2002
Secondary settling tank of a municipal wastewater treatment	Repeated heat treatment	1.96 1.39	Ren <i>et al.</i> , 2008
Sludge compost	No treatment	2.1	Morimoto <i>et al.</i> , 2004
Methanogenic sludge	No treatment	1.51	Wang <i>et al.</i> , 2007
	Heat treatment	1.19	

LA = linoleic acid (C18:2).

\* mol H<sub>2</sub>·mol<sup>-1</sup> glucose

LA enhanced the H<sub>2</sub> production rate and yield in cultures A, B, and C (Table 4.2); this finding is in agreement with findings of Hwu *et al.* (1996) that the 50% inhibitory concentration of LCFAs is independent of the sludge origin. The results also agree with O-Thong *et al.* (2009) that shocking the sludge with high organic load of palm oil mill effluent gave the maximum H<sub>2</sub> production rates in comparison with base, acid, 2-bromoethanesulfonic acid (BESA), and heat shock pretreatments. These researchers reported a 7- and 2-fold increase in H<sub>2</sub> production rate from load-shocked culture in

comparison with the control (untreated) and heat-shocked cultures, respectively. This is in agreement with the results reported in this study because palm oil mill effluent contains 670 to 1800 mg L<sup>-1</sup> of free fatty acids; typical percentage by weight of various fatty acids is: LUA (<1), MA (1-6), PA (32-47), and LA (5-7) (Lim *et al.*, 2005).

The differences observed in the time required to achieve the maximum H<sub>2</sub> yield are likely due to the different kinetics of acidogenic trophic groups under the combined effect of LA and low pH. For example, Chen *et al.* (2005) observed that *Clostridium butyricum* produced H<sub>2</sub> at the end of the exponential growth period. Differences in the types of acidogens present might account for the different response in the municipal suspended acidogenic culture (C) after the first glucose injection in comparison to the industrial granular methanogenic cultures A and B. O-Thong *et al.* (2009) reported that shocking the sludge with palm oil resulted in the dominance of *Thermoanaerobacterium* spp.

The experimental results support the suggestion that a maximum of 4 mol H<sub>2</sub>·mol<sup>-1</sup> glucose is possible from a mixed culture through the acetic/butyric acids fermentation pathway (Kotay and Das, 2009; Cheong and Hansen, 2006). However, they conflict with the conclusions of Kawagoshi *et al.* (2005) that inoculum-conditioning or chemical inhibitors are not always effective in enhancing H<sub>2</sub> yield from all inocula, but depend on inoculums-type and their associated properties. For example, heat and acid treatment efficacy varied depending on the microbial community composition of the mixed culture treated, and this is indicated by the varying H<sub>2</sub> yield in Table 4.8 (from 0.91 to 2.0 mol H<sub>2</sub>·mol<sup>-1</sup> glucose). LA seems to overcome this limitation and maximize H<sub>2</sub> yield despite the differences in the microbial communities, as long as there is sufficient acidogenic activity. In comparison to the reported H<sub>2</sub> yield from glucose in batch mixed culture

studies using other conditioning methods (Table 4.8), LA is more effective in improving H<sub>2</sub> production and increasing the yield (by 48%) than other culture-conditioning methods.

LCFAs in water form micelles, i.e., micro-droplets of water, surrounded by a layer of surfactant molecules. Because both microbial cell surfaces and surfactants are polar, it is most likely that the polar surfaces are surrounded by a thin layer of water. The water layer itself is separated from the fermentation broth medium by a monolayer of surfactant molecules. The surfactant molecules arrange with their polar or ionic head groups towards the water layer and their non-polar part towards the outside; fermentation broth medium (Hoppert *et al.*, 1997). In comparison to aqueous solution, membrane-bound enzymes including hydrogenase in micelles structure have shown improved function. A ten-fold increase in specific activity (turnover rate) was reported by Hoppert *et al.* (1997). This is probably because the water structure inside reversed micelles is very different from free water and that each biological system needs a defined quantity of water in the microemulsion for maximum activity. Hoppert *et al.* (1997) reported that increasing the turnover was based on increased maximal reaction rate or enhanced stability of the enzymes,  $K_m$  values remained unaffected. Enhanced activity or stability of enzymes is considered of major value for use in biotechnological applications. For instance, microemulsions may provide a more favourable surrounding for artificial H<sub>2</sub>-producing systems than aqueous systems Hoppert *et al.* (1997).

Inhibition of methanogens and acetogens by LA is indicated by the lower CH<sub>4</sub> production in LA-treated samples compared to their corresponding controls, and the accumulation of acetate, propionate and butyrate. The increased H<sub>2</sub> yield observed in LA-treated cultures compared to the control cultures may be due to the selective

inhibition of methanogenic activity without the destruction of acidogenic H<sub>2</sub> -producing organisms; while inhibiting methanogens, LCFA maintain a heterogeneous culture. In contrast to this, heat-shock treatment results in a homogeneous and simplified microbial population and reduced H<sub>2</sub> yields (Table 4.8) due to the destruction of other non-spore forming H<sub>2</sub> -producing bacteria (Zhu and Beland, 2006).

The H<sub>2</sub> consumption rates measured in cultures A, B, and C after the first glucose injection were less than the rates reported by Rossetti *et al.* (2003) for anaerobic batch cultures, but very close to those reported by Thiele *et al.* (1988) (Table 4.9). Note that most of the high H<sub>2</sub> consumption rates in Table 4.9, such as the rate of 39.1 d<sup>-1</sup> reported by Shizas *et al.* (2001), were measured at a pH of approximately 7.0.

The greater activity of H<sub>2</sub> degraders in culture B after the first glucose injection is likely due to methanogenic activity. In comparison to cultures A and C, culture B channeled a higher percentage of electron equivalents to methane, both in the controls and LA-treated cultures, particularly after the first glucose injection. The H<sub>2</sub>-consuming activity observed in culture A was likely due to homoacetogenic microorganisms which out-compete methanogens at high H<sub>2</sub> partial pressure conditions (Weijma *et al.*, 2002). This is evident as control samples of culture A channeled 43±8 % of the electron equivalents available in glucose to acetate on Day 10, five days after the second glucose injection, compared to 26±7% for control samples of sludge B. Note that LA-treatment decreased the percentage of electron equivalents used for producing acetate in culture A by 48% (from 43±8% in control samples to 22±1% in LA-treated samples) five days after the second glucose injection. The corresponding reduction for culture B was 27% (from 26±7% in controls to 19±2% in LA-treated samples). H<sub>2</sub> consumption rate decreased in



Table 4.9 Maximum hydrogen specific utilization rates reported for anaerobic consortia.

Culture	Temp (°C)	pH	Substrate	<i>ka</i> ( $\mu\text{mol}\cdot\text{mg}^{-1}$ VSS.day <sup>-1</sup> ) unless indicated otherwise	Reference
Digester sludge	NR*	NR	H <sub>2</sub> /CO <sub>2</sub>	50	Shea <i>et al.</i> , 1968
Primary sludge	37	7.0	H <sub>2</sub> H <sub>2</sub>	44 178	Siegrist <i>et al.</i> , 2002
Domestic wastewater anaerobic sludge	21	7.0	H <sub>2</sub>	39 (d <sup>-1</sup> )	Shizas <i>et al.</i> , 2001
Free flora flocs	37	7.0	H <sub>2</sub>	4 21	Thiele <i>et al.</i> , 1988
Acidic bog sediments	20	4.8 4.2	H <sub>2</sub>	1.4 1.3	Goodwin and Zeikus, 1987
Batch acetogenic culture	NR	6.5	H <sub>2</sub>	1.97	Rossetti <i>et al.</i> , 2003
Digester sludge	30	NR	H <sub>2</sub>	27	Robinson and Tiedje, 1982

\* NR = not reported

LA-treated samples of culture B in comparison to control samples after the first (26%) and second (7%) glucose injections. This was likely due to partial inhibition of homoacetogens in the presence of LA. No data has been published regarding the effects of LCFAs on homoacetogens and further research is required to quantify the effects of LCFAs on homoacetogenesis. The increased rate of H<sub>2</sub> consumption after the second glucose injection in comparison to that observed after the first glucose injection in both control and LA-treated samples is likely due to increased homoacetogenic activity as methanogenesis was completely inhibited. This observation is in agreement with the findings reported by Weijma *et al.* (2002).

The Gompertz model correlates the H<sub>2</sub> production with the experimental data (including the lag phase). Nevertheless, this model does not account for any H<sub>2</sub> consumption due to hydrogenotrophic methanogens after the first glucose injection or homoacetogenic activity after both glucose injections. The latter point is important and has not been addressed in fermentative H<sub>2</sub> production studies.

Accumulation of VFAs reflects a kinetic uncoupling between acidogens/acetogens and methanogens due to the stress imposed by LCFAs (Ahring *et al.*, 1995). VFA accumulation represents the bacteria's response to the stress imposed by H<sub>2</sub> accumulation in the reactor due to the inhibition of methanogens. An important difference between LA-treated cultures A and B is that culture A directed some electron equivalents towards propionate formation, whereas, culture B did not (Table 4.5). Similarly, culture A produced propanol alcohol, whereas, culture B produced ethanol. In addition, LA-treated samples of cultures A and B channeled an increasing percent of electron equivalents to butyrate production and decreased the percent to propanol, isopropanol, and ethanol production compared to their controls. These by-products profile linked to H<sub>2</sub> production are consistent with reported results which showed that butyrate-ethanol type fermentation are preferred for H<sub>2</sub> production compared to propionic acid-type fermentation (Thauer *et al.*, 1977; Ren *et al.* 1997; Cheong and Hansen, 2006). The results indicated that LA induced changes in the metabolic by-products distribution which favored H<sub>2</sub> production.

Applying the PCA to the H<sub>2</sub> yield and metabolic by-products data sets confirmed the differences induced by LA. Control samples of cultures A, B, and C were different from each other, and each was different from its corresponding LA-treated culture. LA-treated cultures clustered together and were very distinct from the control cultures. The loading

maps of the PCA indicated the correlation between the production of H<sub>2</sub> and acetate-butyrate plus propanol formation.

Acidogenic microorganisms synthesize 4 and 3 mol ATP per mol glucose in acetate (Eq. 2.1) and butyrate (Eq. 2.2) production, respectively (Thauer *et al.*, 1977). The increased accumulation of butyrate in LA-treated samples of cultures A and B after the second glucose injection is more advantageous than direct acetogenesis from glucose because less acid is produced per glucose consumed (Goßner *et al.*, 2008). Thauer *et al.* (1977) concluded that the 85% thermodynamic efficiency of the formation of two mol of acetate (Eq. 2.1) is incompatible with the entropy requirements of the microorganisms; therefore, butyrate is always formed and the overall thermodynamic efficiency of the two reactions (Eq. 2.1 and 2.2) is 62% with 3.3 ATP synthesized. The microorganisms in LA-treated cultures in this study demonstrated such a compromise which resulted in higher yields of H<sub>2</sub>.

The FBA model defines the maximum theoretical reference yield of H<sub>2</sub> from glucose taking into account the various metabolites, and then explains the experimental H<sub>2</sub> yield in terms of H<sub>2</sub>-consuming reactions. The MFA model explains the increased H<sub>2</sub> yield in LA-treated samples. The reduction of NAD<sup>+</sup> to NADH<sub>2</sub> competes with the reduction of protons to H<sub>2</sub> (R12) for Fd<sub>red</sub> (Lee *et al.*, 2008). Production of propionate generates NAD<sup>+</sup> by consuming NADH<sub>2</sub> (R6 and R8) and prevents the formation of Fd<sub>red</sub> which is necessary for H<sub>2</sub> production. Therefore, the decrease in propionate production in LA-treated samples of culture A and its complete elimination in culture B mean that less NADH<sub>2</sub> was consumed and less NAD<sup>+</sup> was generated, and this increases the pool of Fd<sub>red</sub> available for the reduction of protons to H<sub>2</sub> (R12). It was suggested that low pH

stimulates the coupling of the reaction of protons with  $\text{Fd}_{\text{red}}$  to form  $\text{H}_2$  (R12) (Saint-Amans *et al.*, 2001). Moreover, Cheong and Hansen (2006) reported that the cell growth rate and biomass yield decreased when the pH was lowered from 5.7 to 4.6 in a  $\text{H}_2$ -producing acidogenic reactor. The pH in the present study is close to that used in Cheong and Hansen's study (2006). The percent electron equivalents spared from cell growth (R2) in this work was likely directed towards  $\text{H}_2$  production (R12) which in turn was stimulated by the low pH due to the accumulation of VFAs. This may explain the increased  $\text{H}_2$  yield after the second glucose injection in LA-treated cultures.

LA reportedly inhibits the degradation of glucose (Alosta *et al.*, 2004; Lalman and Komjarova, 2004). The initial glucose degradation rates in the methanogenic cultures (A and B) were statistically similar, but differed from the acidogenic culture C. LA affected the initial glucose degradation rates in cultures A, B, and C similarly; culture A did not differ significantly from cultures B and C, however, culture C differed significantly from culture B.

The concentration of LA in this work is 10 and 6 times the 50% inhibitory concentration (IC50) reported by Kim *et al.* (2004) for acetate and propionate degradation, respectively, and 20 and 2 times the concentration of LA which inhibited butyrate degradation in glucose and butyrate adapted mixed cultures, respectively (Lalman and Bagley, 2002; Mykhaylovin *et al.*, 2005). Inhibition of acetoclastic methanogens correlated with increased LCFAs concentration (Shin *et al.*, 2003). Moreover, pH 5 in this study is lower than that in the studies cited, and there are synergistic effects with other VFAs produced from glucose in this study. Acetate accumulated because LA inhibited the acetoclastic methanogens. LA is inhibitory to

butyrate and propionate degrading acetogenic bacteria (Lalman and Bagley, 2002; Kim *et al.*, 2004; Mykhaylovin *et al.*, 2005). Butyrate and propionate also accumulate because their degradation to acetate is thermodynamically impossible under the high H<sub>2</sub> partial pressure and LA-inhibited methanogenesis ( $\Delta G^{\circ} = + 48.30$ , and  $+71.67$  kJ/mol, respectively) (McCarty and Smith, 1986). Kasper and Wuhrmann (1978) reported that propionate degradation was completely stopped by high concentrations (500-5000 ppm) of H<sub>2</sub> in the digester gas. However, some propionate was degraded in LA-treated samples of culture A between Day 5 and Day 10 of the fermentation. Although propionate degradation was previously reported to occur despite a very high H<sub>2</sub> concentration, its degradation in the presence of LA in this work is likely due to the inhomogeneous conditions in the environment and microbial granules (Krylova and Conrad, 1998). The local mini and the bulk environments could be different.

If about 66% of the electron equivalents in glucose are used in only VFAs with limited growth, then the remaining 33% of the electron equivalents could be recovered as H<sub>2</sub>. The electron mass balance indicated that H<sub>2</sub> accounted for approximately 26% of the electrons that could be recovered as H<sub>2</sub>. This represents recovery efficiency of 78%. LA seems to increase the H<sub>2</sub> yield via minimizing its consumption; however, the results of this study indicated the need for more research on ways to control homoacetogenic H<sub>2</sub> consumption during H<sub>2</sub> fermentation.

## 4.5 CONCLUSIONS

1. Treatment with LA enhanced H<sub>2</sub> production from glucose in anaerobic mixed cultures from three different sources under mesophilic low pH conditions; the maximum H<sub>2</sub> yield obtained was 3.11 mol H<sub>2</sub>·mol<sup>-1</sup> glucose. LA overcame the

differences among cultures from different sources and increased the H<sub>2</sub> yield to comparable levels in all cultures.

2. LA-treatment inhibited methane production completely, and shifted the pathway towards reduced propionate and increased butyrate production in the different cultures. Adding LA directed greater proportions of electron equivalents from glucose towards H<sub>2</sub> and away from other reduced by-products. LA favors Acetate-butyrate and propanol formation was favorable in the presence of LA and this resulted in increasing the H<sub>2</sub> yield.
3. LA-treatment seems to overcome the limitations associated with other H<sub>2</sub>-producing enrichment methods, and maximized H<sub>2</sub> production despite differences in the amount and activity of the various trophic groups.
4. Hydrogen consumption proceeded via homoacetogenesis from H<sub>2</sub>/CO<sub>2</sub>, but it was effectively reduced by LA.
5. PCA is a useful tool to compare different cultures using the data sets of metabolic by-products. Concurrently, the FBA explained the H<sub>2</sub>-production and H<sub>2</sub>-consumption reactions depended on the measured extracellular by-products.

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## **CHAPTER 5: EFFECTS OF ADAPTATION TO GLUCOSE ON MIXED CULTURE COMPOSITION, ACTIVITY, AND HYDROGEN PRODUCTION**

### **5.1 INTRODUCTION**

Normally, microorganisms are grown under controlled conditions in the laboratory to enhance their growth. This approach has been used to define the effects of inocula source on hydrogen (H<sub>2</sub>) production. However, laboratory growth conditions and enrichment have severe limitations and impose selective pressures which act against or with most microorganisms. This process screens out many fastidious organisms (e.g., methanogens) as well as organisms that depend on close interactions with other species for their survival (syntrophs). Not all microorganisms from a particular environment can be grown in the laboratory (Amann *et al.*, 1995). This inability to grow most of the microorganisms in the actual consortium from a particular environment raises the question whether the laboratory-cultivated cultures represent their source environment or not?

Hydrogen production has been reported in cultures from different sources in laboratory studies with different periods of acclimatizing the culture to the substrate, new temperature, growth media, or other environmental conditions. No attention has been given to potential changes in cultures during storage or adaptation to the laboratory conditions that might affect H<sub>2</sub> yield.

The bacterial populations depend on the media used (Sørheim *et al.*, 1989) and maintenance procedures. For example, the nutrient-rich media used in laboratory cultivation introduces serious bias to the microbial community (Boivin-Jahns, 1995;

Ferris *et al.*, 1996). The selection might be biased towards copiotrophic bacteria rather than dominant community members (Angert, 1998). Moreover, laboratory-grown microorganisms lack interaction with other populations and with their natural environment. Laboratory-maintained cultures cannot represent the diversity of their original cultures within their natural environments (NSF *et al.*, 1999) because adaptation to the laboratory environment and stringent nutrient limitations induce physiological and phylogenetic changes in the microbial community (LaPara *et al.*, 2006). The physiological transformation includes alterations in the enzymatic level, regulation and production, mutations, etc (Wiggings *et al.*, 1987). Therefore, the cultures need to be defined physiologically and phylogenetically before use in H<sub>2</sub> production studies and their performance needs to be related to their composition.

Microbial populations adapt to stressful changes in conditions such as non-optimal temperature and pH, concentrations of ions, substrates, nutrients, and presence of toxins by developing reversible or irreversible alterations in the genetic information of the cellular components (van Veen *et al.*, 1997). Environmental changes can induce expression or repression of genes in bacteria, and changes in the properties of internal and external surface chemistry of cell membranes. Such genetic changes are maintained and passed to progeny. Furthermore, stressful conditions increase the probability of erroneous DNA synthesis and impairment of DNA-repair systems (Kivisaar, 2003). The genetic aspects of adaptation might become an important area of research in H<sub>2</sub> production.

Acclimation to a new substrate results in selection and multiplication of specialized microorganisms because the substrate-tolerant microorganisms dominate over the substrate-sensitive ones (Novaes, 1986). The ability of a mixed culture to treat various

substrates such as carbohydrates or “intermediate” products of anaerobic metabolism such as lactic, propionic, butyric, and acetic acids characterize these changes (Gavala and Lyberatos, 2001; Hattingh *et al.*, 1967; Guieysse *et al.*, 2001). For example, types of methanogenic species and their relative population levels in a reactor depends on the wastewater characteristics as well as on the operational and environmental conditions (Novaes, 1986; Jawed and Tare, 1999). The activities of the various trophic groups in a mixed culture is affected by substrate acclimatization. Dolfing and Bloemen (1985) reported that the presence of 10% sucrose in the growth medium reduced the methanogenic activity of the biomass by 30-70% in comparison to the activity of the biomass cultivated on acetate and propionate. The methanogenic activities of the biomass after three months of cultivation reflected the composition of the growth medium rather than the activities of the inoculum (Dolfing and Bloemen, 1985). A very active methanogenic culture lost its methane production activity completely after 2-3 years of enrichment on butanol (Yu *et al.*, 2005; Yu and Semprini, 2002). The maximum specific utilization rates of various compounds and the stoichiometry of volatile fatty acids production changed significantly after 18 months of adaptation to lactose and gelatin (Gavala and Lyberatos, 2001).

Biomass acclimatization is important factor to increase H<sub>2</sub> production and substrate consumption (Abreu *et al.*, 2009). Synthesizing an efficient H<sub>2</sub>-producing “design” consortium initially requires defining it physiologically and phylogenetically. The first step is to characterize the consortia physiologically, or functionally, in order to define its composition in terms of the various active trophic groups and their metabolic activities. Concurrently, the consortia have to be characterized phylogenetically using molecular

biology methods with proper resolution. Integrating kinetic, molecular biology, and statistical methods produces useful information about the dynamics and activity of H<sub>2</sub>-producing anaerobic mixed cultures (Guieysse *et al.*, 2001; Blagodatsky *et al.*, 2000).

The objective of this research is to use a combination of kinetic, molecular biology, and statistical methods to define the effects of long-term adaptation to glucose on the structure, composition, and activity of H<sub>2</sub>-producing mixed anaerobic cultures. The experimental work quantifies the presence of individual active trophic groups and their activities within three anaerobic cultures from different sources before and after one year of adaptation to glucose. Glucose-non-acclimatized and glucose-acclimatized samples of the same three cultures were characterized kinetically to determine the effects of substrate acclimatization and laboratory maintenance conditions on composition, activity, and functioning of these cultures.

## **5.2 METHODS AND MATERIALS**

Inocula sources and maintenance of the cultures used in this work are described in section 3.1, the experimental design in section 3.4.2.1, and the analytical methods in section 3.5. Kinetic characterization was conducted in accordance with the protocol described in section 3.3 and the calculations followed the procedure given in Appendix C. A sample calculation for a single culture is given in Appendix C.

## **5.3 RESULTS**

### **5.3.1 Kinetics of glucose-adapted and non-adapted cultures**

Substrate degradation time curve assays were performed with glucose, lactate, acetate, propionate, butyrate, and H<sub>2</sub> to determine the maximum specific substrate utilization rates

and the percentage of active specific substrate degraders for cultures A, B and C before and after one year of adaptation to glucose in laboratory conditions. The substrate degradation time curves are shown in Appendix C. The maximum specific substrate utilization rate ( $k_a$ ) and percentage of biomass concentration of the active trophic group ( $X_a$ ) were determined for each substrate (Table 5.1 and Figure 5.1). Mixed anaerobic cultures were procured from industrial and municipal treatment facilities. Methanogenic granular cultures A and B were obtained from UASB reactors treating effluents from a brewery and ethanol manufacturing plant. A suspended/flocculent culture designated as C was obtained from an anaerobic digester in a municipal wastewater treatment facility.

Statistical comparison of the specific activities of the various trophic groups in culture A before and after adaptation to glucose indicated that only lactate and propionate maximum utilization rates before adaptation to glucose were significantly different from those after adaptation. The corresponding percentages of the biomass concentration for various trophic groups were similar.

Statistical comparison of the specific activities of culture B indicated that the maximum substrate utilization rates before adaptation to glucose were similar to those after adaptation. However, the corresponding percentages of the biomass concentration for the various trophic groups were significantly different except for butyrate and propionate. The total active biomass differed significantly. A statistical comparison of the specific activities of culture C indicated that the maximum substrate utilization rates before adaptation to glucose were statistically similar to those after adaptation. The percentages of the biomass concentration for the various trophic groups were similar



Table 5.1 Maximum specific substrate utilization rates and biomass concentration (percentage) of individual active trophic groups in glucose-adapted and non-adapted cultures (from cultures A, B, and C; mean±standard deviation of triplicate samples).

Substrate	Culture A			
	$k_a$ (d <sup>-1</sup> )		$X_a$ (%)	
	Adapted	Non-adapted	Adapted	Non-adapted
Glucose	23.07±4.77 <sup>a</sup>	16.28±5.59 <sup>a</sup>	14.57±5.03 <sup>a</sup>	8.79±2.21 <sup>a</sup>
Lactate	283.79±17.69 <sup>a</sup>	99.03±41.25 <sup>b</sup>	0.58±0.34 <sup>a</sup>	2.98±1.42 <sup>a</sup>
Acetate	10.97±8.99 <sup>a</sup>	4.44±0.54 <sup>a</sup>	3.43±3.63 <sup>a</sup>	1.85±0.28 <sup>a</sup>
Butyrate	16.04±7.68 <sup>a</sup>	17.81±1.19 <sup>a</sup>	1.03±0.65 <sup>a</sup>	0.93±0.36 <sup>a</sup>
Propionate	1.2±0.69 <sup>a</sup>	3.31±0.59 <sup>b</sup>	5.17±3.38 <sup>a</sup>	3.37±1.36 <sup>a</sup>
Hydrogen	8.2±5.85 <sup>a</sup>	28.96±14.39 <sup>a</sup>	10.67±6.08 <sup>a</sup>	5.03±1.88 <sup>a</sup>
Total active biomass			35.45±8.4 <sup>a</sup>	22.95±1.64 <sup>a</sup>

Substrate	Culture B			
	$k_a$ (d <sup>-1</sup> )		$X_a$ (%)	
	Adapted	Non-adapted	Adapted	Non-adapted
Glucose	10.40±1.25 <sup>a</sup>	12.79±0.26 <sup>a</sup>	11.19±2.31 <sup>a</sup>	7.32±2.69 <sup>b</sup>
Lactate	31.13±5.83 <sup>a</sup>	44.62±30.26 <sup>a</sup>	1.56±0.71 <sup>a</sup>	3.46±0.81 <sup>b</sup>
Acetate	1.65±0.78 <sup>a</sup>	6.96±2.63 <sup>a</sup>	6.91±1.87 <sup>a</sup>	0.60±0.17 <sup>b</sup>
Butyrate	1.90±0.35 <sup>a</sup>	5.80±1.57 <sup>a</sup>	2.31±0.76 <sup>a</sup>	1.10±0.14 <sup>a</sup>
Propionate	3.16±0.68 <sup>a</sup>	5.27±2.4 <sup>a</sup>	0.89±0.28	1.04±0.64
Hydrogen	20.56±2.85 <sup>a</sup>	45.90±17.37 <sup>a</sup>	4.15±1.27 <sup>a</sup>	0.63±0.43 <sup>b</sup>
Total active biomass			27.01±3.93 <sup>a</sup>	14.15±1.21 <sup>b</sup>

Substrate	Culture C			
	$k_a$ (d <sup>-1</sup> )		$X_a$ (%)	
	Adapted	Non-adapted	Adapted	Non-adapted
Glucose	39.78±10.87 <sup>a</sup>	29.52±10.42 <sup>a</sup>	12.23±2.79	10.50±2.01
Lactate	59.71±4.09 <sup>a</sup>	251.5±49.09 <sup>a</sup>	6.43±0.57 <sup>a</sup>	0.98±0.38 <sup>b</sup>
Acetate	8.02±2.87 <sup>a</sup>	5.74±1.67 <sup>a</sup>	2.77±1.53 <sup>a</sup>	4.91±1.16 <sup>a</sup>
Butyrate	6.16±0.65 <sup>a</sup>	10.3±1.18 <sup>a</sup>	3.86±0.7 <sup>a</sup>	3.58±0.84 <sup>a</sup>
Propionate	7.53±0.73 <sup>a</sup>	17.17±7.35 <sup>a</sup>	3.42±0.53 <sup>a</sup>	1.23±0.48 <sup>b</sup>
Hydrogen	30.94±2.5 <sup>a</sup>	66.0±25.42 <sup>a</sup>	2.59±0.77 <sup>a</sup>	1.06±0.63 <sup>a</sup>
Total active biomass			31.30±3.48 <sup>a</sup>	22.26±2.91 <sup>b</sup>

$k_a$  = maximum specific substrate utilization rate (mg substrate as COD mg<sup>-1</sup> VSS as (COD d<sup>-1</sup>)  
 $X_a$  = percentage of the biomass concentration for specific substrate degraders (%). Note: <sup>a</sup> and <sup>b</sup> indicate significantly different means of  $k_a$  or  $X_a$  in the same row. No comparison should be made between rows.

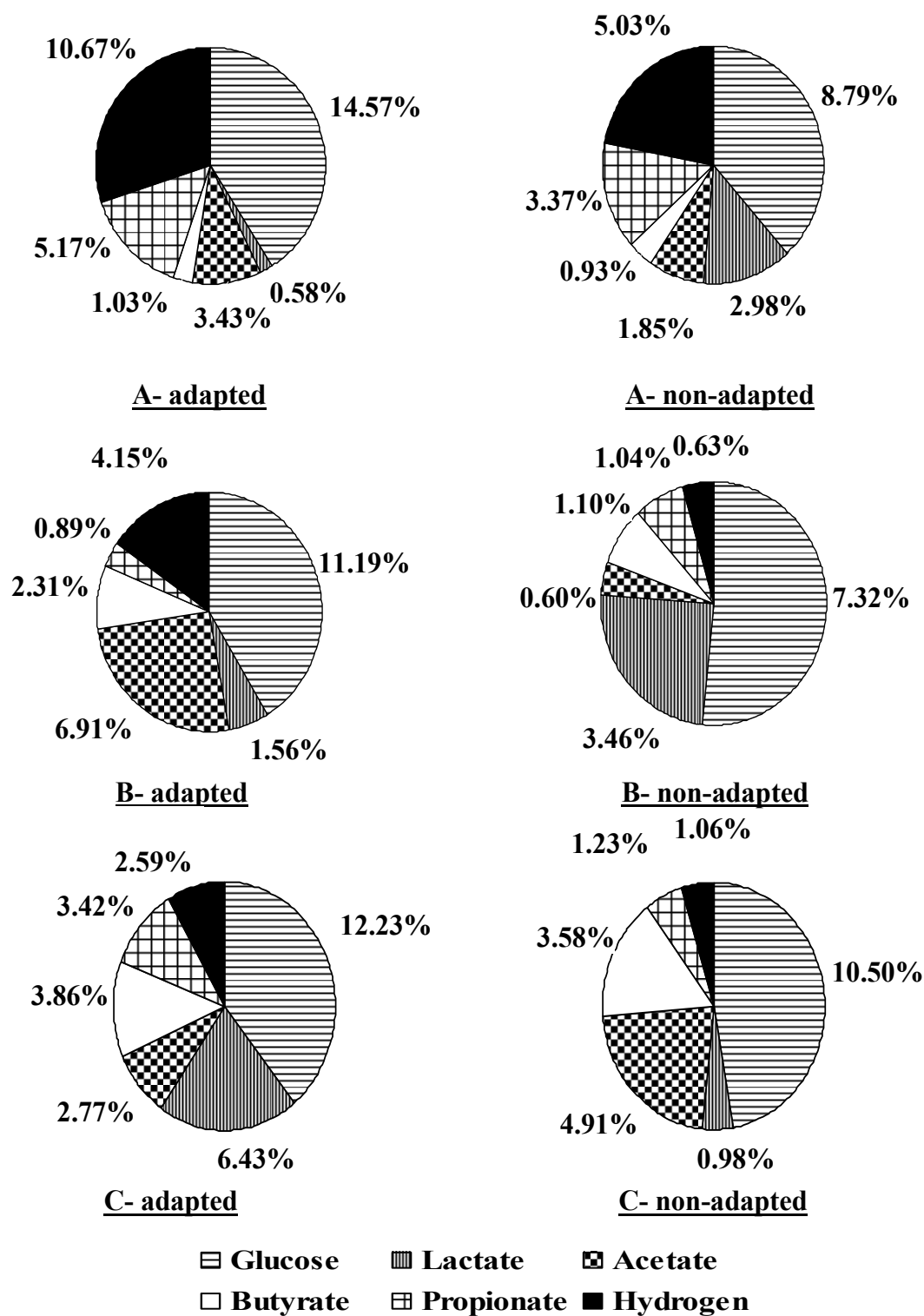


Figure 5.1 Composition of culture A, B, and C before and after 12 months of adaptation to glucose in laboratory conditions. (The percentage is based on the total biomass).

except for the lactate and propionate degraders. The total active biomass was also significantly different. The p-values (two-tailed t-test) of the maximum specific substrate utilization rates and the percentage of the biomass concentration of the active trophic groups for culture A, B, and C before and after adaptation to glucose are given in Table 5.2.

Table 5.2 p-values for comparison of means of adapted and non-adapted cultures using two-tailed t-test.

<u>Substrate</u>	<u>Culture A</u>		<u>Culture B</u>		<u>Culture C</u>	
	$k_a$	$X_a$	$k_a$	$X_a$	$k_a$	$X_a$
<b>Glucose</b>	0.508	0.087	0.062	<b>0.029</b>	0.934	0.162
<b>Lactate</b>	<b>0.006</b>	0.107	0.527	<b>0.038</b>	0.068	<b>0.0001</b>
<b>Acetate</b>	0.216	0.532	0.079	<b>0.028</b>	0.320	0.125
<b>Butyrate</b>	0.732	0.827	0.104	0.056	<b>0.013</b>	0.678
<b>Propionate</b>	<b>0.016</b>	0.456	0.279	0.729	0.152	<b>0.006</b>
<b>Hydrogen</b>	0.104	0.264	0.130	<b>0.045</b>	0.140	0.055

Note: The units:  $k_a = (\text{d}^{-1})$ ;  $X_a = (\%)$ . P-values in bold font indicate significant difference.

Similar changes in biomass concentration were observed in all cultures for glucose- and butyrate-degrading organisms as well as hydrogen-consumers; these trophic groups increased in all cultures after adaptation. The increase in acidogenic biomass contributed to the increase in the total active biomass. Cultures A and B differed from Culture C as lactate-degraders decreased and acetate-degraders increased. Culture B differed from Cultures A and C, however, as propionate-degraders decreased in B, but increased in A and C. Table 5.3 illustrates the contrasting responses for the cultures; decreased biomass concentration percentages are indicated by negative values.

The decrease in the percentage of lactate-degraders in culture B after adaptation was associated with a decrease in the percentage of propionate-degrading microorganisms, while the opposite was observed in culture C; the 560% increase in the percentage of

lactate-degraders in culture C after adaptation corresponded to the 180% increase in the percentage of propionate-degrading microorganisms. The three cultures all showed increased percentages of glucose-, H<sub>2</sub>- and butyrate-consuming microorganisms though the degree of the increase varied among cultures.

Table 5.3 Change in the percentage biomass concentration and activities of the various trophic groups due to long-term adaptation to glucose.

<u>Substrate</u>	<u>Percentage of change after adaptation in</u>					
	<u><math>k_a</math></u>			<u><math>X_a</math></u>		
	<u>Maximum specific</u>			<u>Active biomass</u>		
	<u>substrate utilization rate</u>			<u>Culture</u>		
	<u>Culture</u>			<u>Culture</u>		
	<u>A</u>	<u>B</u>	<u>C</u>	<u>A</u>	<u>B</u>	<u>C</u>
<b>Glucose</b>	41.70	-18.68	34.76	65.76	52.87	16.48
<b>Lactate</b>	186.5	-30.23	-76.26	-80.54	-54.91	556.1
<b>Acetate</b>	147.07	-76.29	39.72	85.41	1051.6	-43.58
<b>Butyrate</b>	-9.94	-67.24	-40.19	10.75	110.0	7.82
<b>Propionate</b>	-63.75	-40.04	-56.14	53.41	-14.42	178.0
<b>Hydrogen</b>	-71.69	-55.21	-53.12	112.1	558.7	144.3
<b>Total active biomass</b>				54.47	95.30	40.61

Note:

- Culture A is methanogenic granular culture from a UASB reactor treating brewery wastewater.
- Culture B is methanogenic granular culture from a UASB in ethanol manufacturing facility.
- Culture B is acidogenic/methanogenic suspended/flocculent culture from an anaerobic digester in a municipal wastewater treatment facility.

The increase or decrease in the percent of the various active biomass concentration reflects the effects of the enriched growth media, and the stresses imposed by the maintenance and operation conditions in the laboratory.

The activities of glucose-degrading microorganisms increased in all cultures after adaptation. The maximum specific substrate utilization rates of lactate and acetate in culture A and acetate in culture C increased after adaptation whereas the activities of all other trophic groups decreased. The magnitude of the decrease in the maximum specific

substrate utilization rates varied among cultures. The lactate-degrading activity of cultures B and C decreased by 30 and 76%, respectively (Table 5.3). The decrease in the maximum specific utilization rates of acetate, butyrate and  $H_2$  ranged between 10 and 70% in all cultures. The activity of  $H_2$  -consumers decreased in all cultures after adaptation. The maximum  $H_2$ -specific utilization rate decreased by 72, 55, and 53% in cultures A, B, and C, respectively.

It is evident that the changes in the percentages of the biomass concentration of the trophic groups due to adaptation to glucose did not correlate with the changes in their maximum specific substrate utilization rates;. A comparison of the pattern of the shaded cells denoting a decrease in the utilization rates or biomass concentration for the substrates is shown in Table 5.3. This might be due to the redundancy and mixotrophic nature in microbial functions of the various trophic groups involved in the mixed cultures.

### **5.3.2 Hydrogen production before and after adaptation to glucose**

The results of the  $H_2$  production experiments in control and LA-treated samples of glucose-adapted cultures are shown in Table 5.4. The maximum  $H_2$  yield after the first and second glucose injection are shown in bold font cast. For comparison purposes, the  $H_2$  yields in phase II were shown with the  $H_2$  yields of the non-adapted cultures from the  $H_2$  production experiment in phase I (Figure 5.2). The  $H_2$  yields for the glucose-adapted cultures were higher than the yields in non-adapted cultures after the first and second glucose injections. After the second glucose injection, non-adapted cultures exhibited greater  $H_2$  consumption compared to glucose-adapted cultures (Figure 5.2). However, LA increased the yields of  $H_2$  in both glucose-adapted and non-adapted cultures to

comparable levels, and most importantly, decreased the rate of subsequent H<sub>2</sub> consumption in the adapted cultures more than in the non-adapted cultures (Table 5.5).

Table 5.4 Hydrogen yield in glucose-adapted cultures (A, B, and C) (cultures fed only 5,000 mg L<sup>-1</sup> glucose and 5,000 mg L<sup>-1</sup> glucose plus 2,000 mg L<sup>-1</sup> LA (glucose added at day 0 and day 5; mean±standard deviation for triplicate samples).

<b>Hydrogen yield in adapted culture (mol H<sub>2</sub>·mol<sup>-1</sup> glucose)</b>						
<b>Day</b>	<b>Culture A</b>		<b>Culture B</b>		<b>Culture C</b>	
	<b>AC</b>	<b>ALA</b>	<b>BC</b>	<b>BLA</b>	<b>CC</b>	<b>CLA</b>
<b>1</b>	0.80±0.10	1.46±0.22	0.24±0.07	0.38±0.20	0.50±0.15	1.13±0.05
<b>2</b>	1.69±0.28	2.36±0.16	0.59±0.34	1.84±0.00	1.09±0.26	1.68±0.00
<b>3</b>	<b>1.82±0.29</b>	2.64±0.45	1.13±0.46	2.80±0.64	2.29±0.35	1.92±0.08
<b>4</b>	1.58±0.16	<b>2.74±0.10</b>	<b>1.63±0.60</b>	<b>2.96±0.31</b>	<b>2.34±0.54</b>	<b>2.62±0.47</b>
<b>5</b>	0.13±0.14	0.55±0.25	0.68±0.17	0.89±0.22	1.08±0.36	1.04±0.29
<b>6</b>	0.37±0.37	1.16±0.11	1.65±0.43	1.87±0.05	1.55±0.26	1.50±0.38
<b>7</b>	0.61±0.62	2.00±0.07	<b>2.05±0.69</b>	2.96±0.08	1.58±0.30	1.93±0.52
<b>8</b>	<b>1.67±0.72</b>	<b>2.46±0.11</b>	2.05±0.30	<b>3.32±0.12</b>	<b>1.65±0.36</b>	<b>2.48±0.17</b>

Note: numbers in bold font indicate the maximum yields after the first and the second glucose injections.

Table 5.5 Hydrogen-specific consumption rate during H<sub>2</sub> production experiments (mean±standard deviation for triplicate samples).

<b>Glucose Injection Number</b>	<b>Culture</b>	<b>H<sub>2</sub> consumption rate (μmol H<sub>2</sub> mg VSS<sup>-1</sup> day<sup>-1</sup>)</b>			
		<b>Control</b>		<b>2,000 mg L<sup>-1</sup> LA</b>	
		<b>Adapted</b>	<b>Non-adapted</b>	<b>Adapted</b>	<b>Non-adapted</b>
<b>1</b>	<b>A</b>	0.19±0.04 <sup>a</sup>	0.0±0.0 <sup>b</sup>	0.06±0.10 <sup>a</sup>	0.0±0.0 <sup>a</sup>
	<b>B</b>	0.0±0.0 <sup>a</sup>	1.95±0.99 <sup>a</sup>	0.11±0.09 <sup>a</sup>	1.44±0.89 <sup>a</sup>
	<b>C</b>	0.18±0.16 <sup>a</sup>	0.83±0.12 <sup>b</sup>	0.27±0.05 <sup>a</sup>	0.31±0.21 <sup>a</sup>
<b>2</b>	<b>A</b>	0.0±0.0 <sup>a</sup>	0.0±0.0 <sup>a</sup>	0.0±0.0 <sup>a</sup>	1.73±1.45 <sup>a</sup>
	<b>B</b>	0.14±0.24 <sup>a</sup>	2.96±1.95 <sup>a</sup>	0.0±0.0 <sup>a</sup>	2.11±0.68 <sup>b</sup>
	<b>C</b>	0.0±0.0 <sup>a</sup>	3.27±1.75 <sup>a</sup>	0.0±0.0 <sup>a</sup>	2.58±1.49 <sup>a</sup>

Note: <sup>a</sup> and <sup>b</sup> indicate significantly different means of adapted and non-adapted cultures. No comparison between different rows should be made.

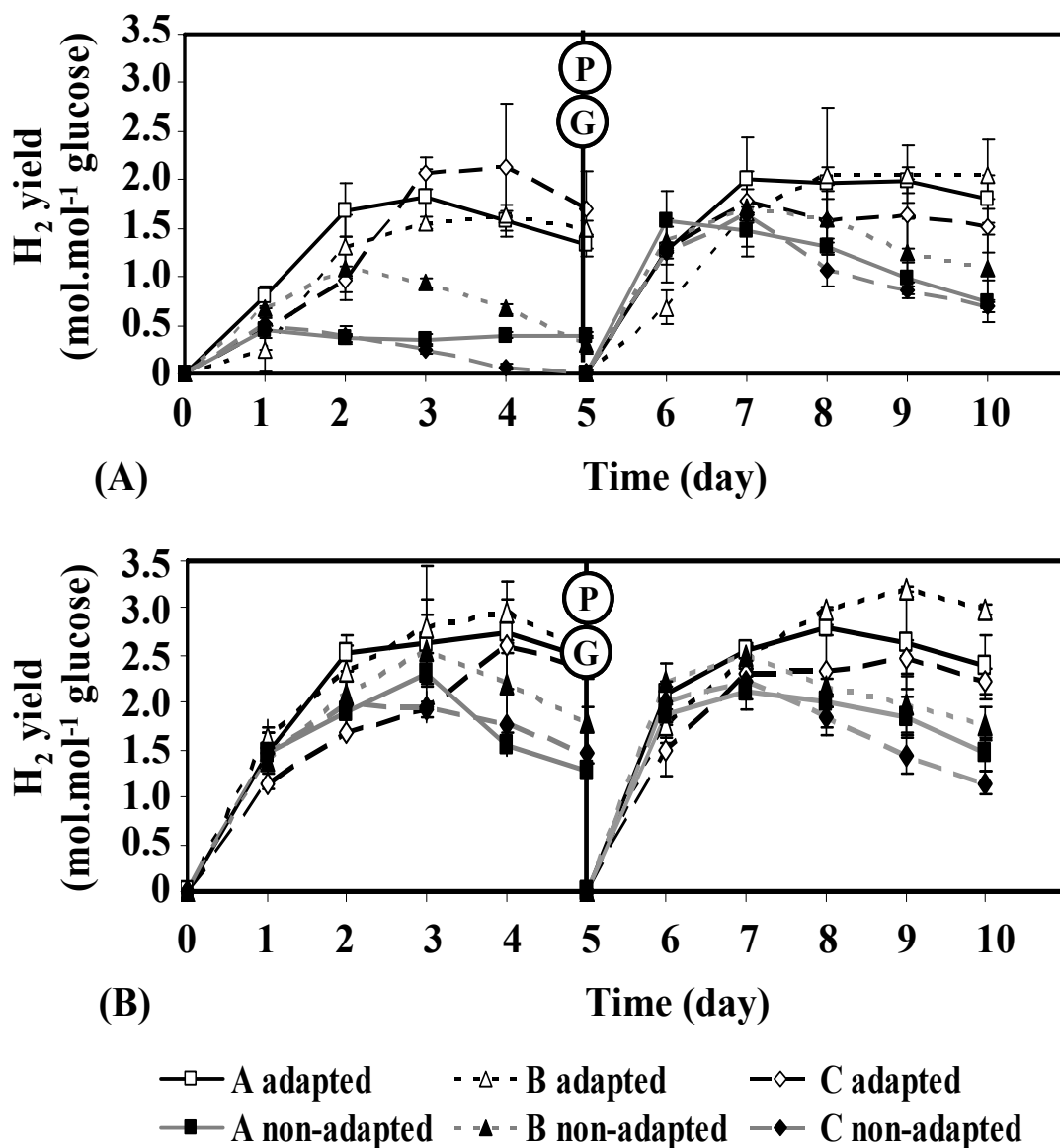


Figure 5.2 Hydrogen yield from culture A, B, and C before and after adaptation to glucose for 12 months; effect of LA-treatment. All cultures fed 5,000 mg L<sup>-1</sup> glucose. (A) Glucose control samples, (B) Glucose plus 2,000 mg L<sup>-1</sup> LA samples. (Mean±standard deviation for triplicate samples).

### 5.3.3 Principal component analysis (PCA)

The difference between the glucose-adapted and non-adapted cultures was confirmed using Principal Component Analysis (PCA). Two analyses were conducted using three data sets. The first included the maximum specific substrate utilization rates ( $k_a$ ); the

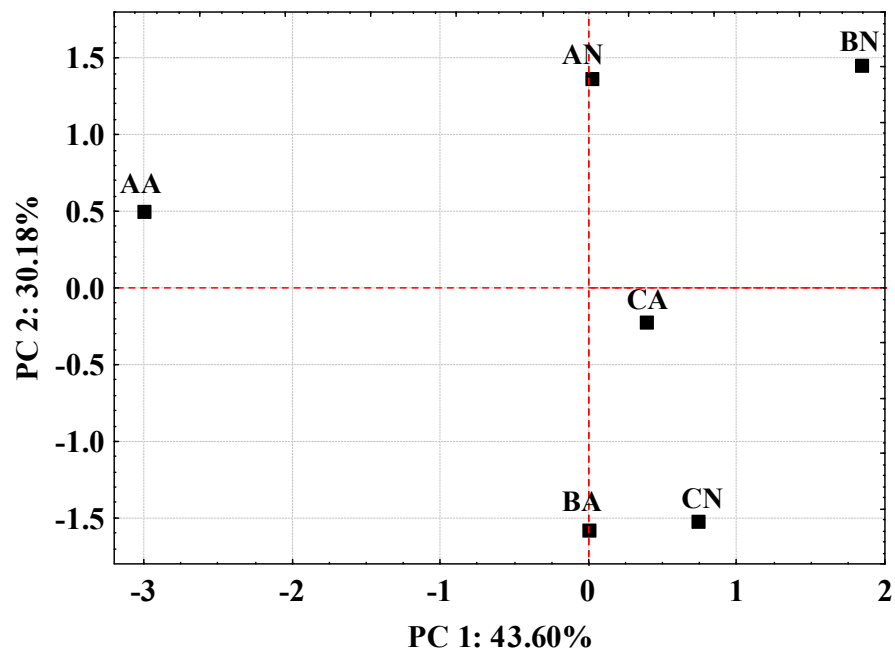
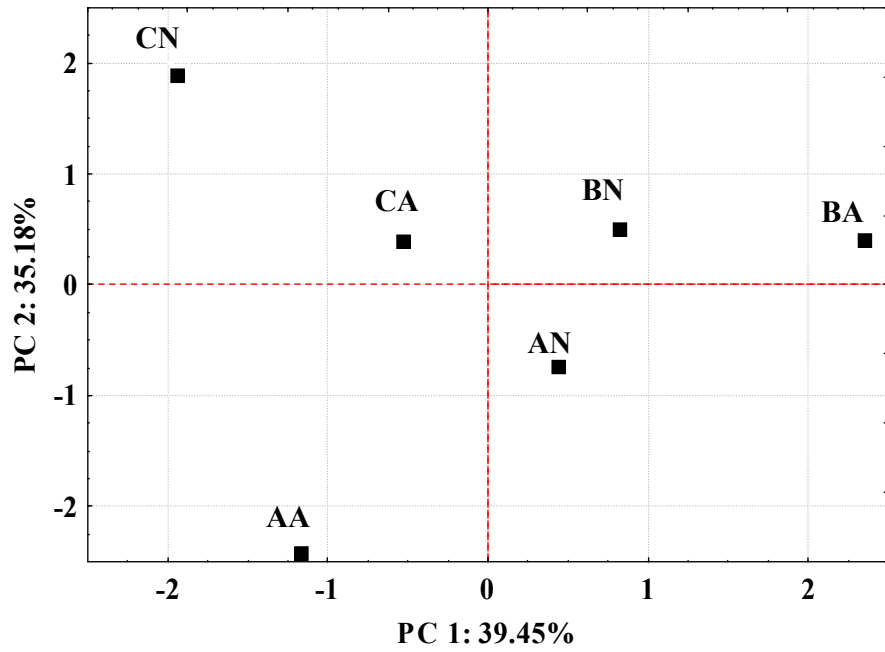
second included the percentage of the biomass concentration of the active trophic groups ( $X_a$ ); and the third data set combined the previous two data sets ( $k_a$  and  $X_a$ ). The data set comprised of the  $H_2$  yields from cultures A, B, and C before and after 12 months of adaptation to glucose was also subjected to PCA. The score map reveals groups by clustering, trends by changing locations, or similarities/differences among samples.

The PCA score maps for the first three data sets, the kinetic parameters, are shown in Figure 5.3. The first two principal components explained 75% of the variation in the  $k_a$  data set. PC1 and PC2 accounted for 39 and 35% of the variation in the data, respectively (Figure 5.3A). In the PCA of the second data set, the first two principal components explained 74% of the variation in ( $X_a$ ) data set. PC1 and PC2 accounted for 44 and 30% of the variation in the data, respectively (Figure 5.3B). In the PCA of the combined data sets of  $k_a$  and  $X_a$ , the first two principal components explained 62% of the variation in the data set. PC1 and PC2 accounted for 38 and 25% of the variation in the data, respectively (Figure 5.3C). The difference between the glucose-adapted and non-adapted cultures is quite clear since for each culture (A, B, and C), the glucose-adapted culture was located far from its corresponding non-adapted culture on the map of their projections on the PC1 and PC2 axes, however, no clustering was observed. Thus, these analyses did not establish similarity within either the domain of glucose-adapted cultures or the domain of non-adapted cultures. Therefore, it can be concluded that cultures A, B, and C were kinetically different from each other both before and after glucose-adaptation.

PCA was also applied to the  $H_2$  yield data set (Figure 5.4), notice the close association and clustering of the three cultures (A, B, and C) for each condition (control and LA-treated samples before and after glucose-adaptation). Four clusters clearly isolate the



three cultures (A, B, and C) within each experimental condition on the score map of PC1-PC2.



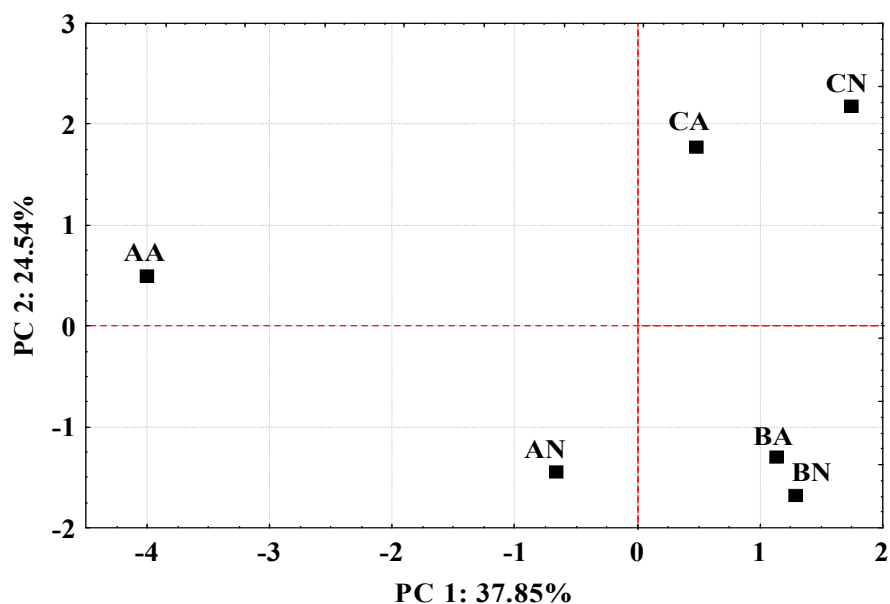


Figure 5.3 PCA for the data sets of (A) maximum specific substrate utilization rates, (B) percentages of biomass concentration of the active trophic groups, (C) combined data sets of (A) and (B) for culture designated as A, B, and C before and after 12 months of adaptation to glucose. (AA, BA, and CA= culture A, B, and C adapted to glucose, respectively. AN, BN, and CN= culture A, B, and C non-adapted to glucose, respectively.)

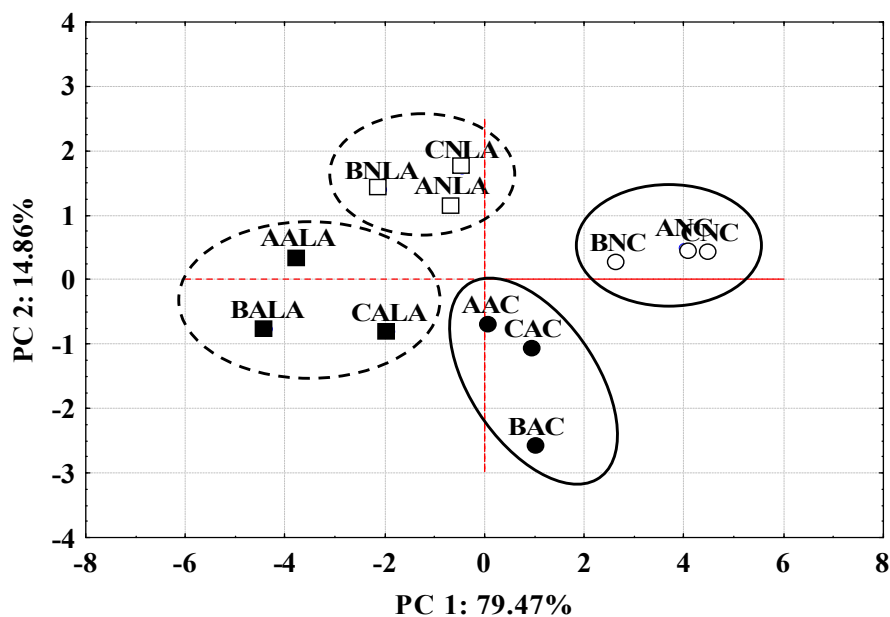


Figure 5.4 PCA for the  $H_2$  yield data set for cultures A, B, and C before and after 12 months of adaptation to glucose. (AAC, BAC, and CAC= control samples of culture A, B, and C adapted to glucose. ANC, BNC, and CNC= control samples of culture A, B, and C non-adapted to glucose. AALA, BALA, and CALA= LA-fed samples of culture A, B, and C adapted to glucose. ANLAC, BNLA, and CNLA = LA-fed samples of culture A, B, and C non-adapted to glucose).

### 5.3.4 Microbiological analysis

T-RFLP was used to characterize cultures A, B, and C microbiologically before and after 12 months of adaptation to glucose according to the protocol described in section 3.6. The size range of most T-RFs was between 50 and 565 bp. Many (106) T-RF's were detected with *Hae* III, *Hha* I and *Msp* I enzymes in the non-adapted samples, whereas only 67 T-RF's were detected in glucose-adapted culture samples (Figure 5.5).

The relative area was used to estimate the relative abundance (percentage) of the species represented by each band. The relative abundance is plotted as percentage against the corresponding fragment size (bp) detected for the enzyme *Hae* III in Figure 5.6.

The Shannon diversity index (Table 5.6) was calculated to compare the diversity of cultures A, B, and C before and after adaptation to glucose. The results indicate that the diversity of the three cultures decreased after 12 months of glucose-adaptation. Adaptation affected the diversity of culture A and C more than culture B, the least affected. The number of the dominating bands in culture A and C after adaptation to glucose is less than before adaptation (Figure 5.5).

Glucose-adapted samples of culture A included fewer dominating bands (5 T-RF's) than the non-adapted samples of this culture (14 T-RF's) (Figure 5.5A). Before adaptation to glucose, culture A contained [lane b in Figure 5.5 (A) and Figure 5.6 (A)] *Fusobacterium* (band 6) 24 %; *Bacteroides*, *Flavobacterium*, *Acinetobacter*, and *Syntrophus* (band 4) 19%; *Brevibacillus* and *Butyrivibrio* (band 8) (10%); *Geobacter* and *Acidimicrobium* (band 9) 7%; *Parabacteroides* (band 13) (3%); *Thermoanaerobacter* (band 1) 2%) while *Clostridia* formed less than 1%. After

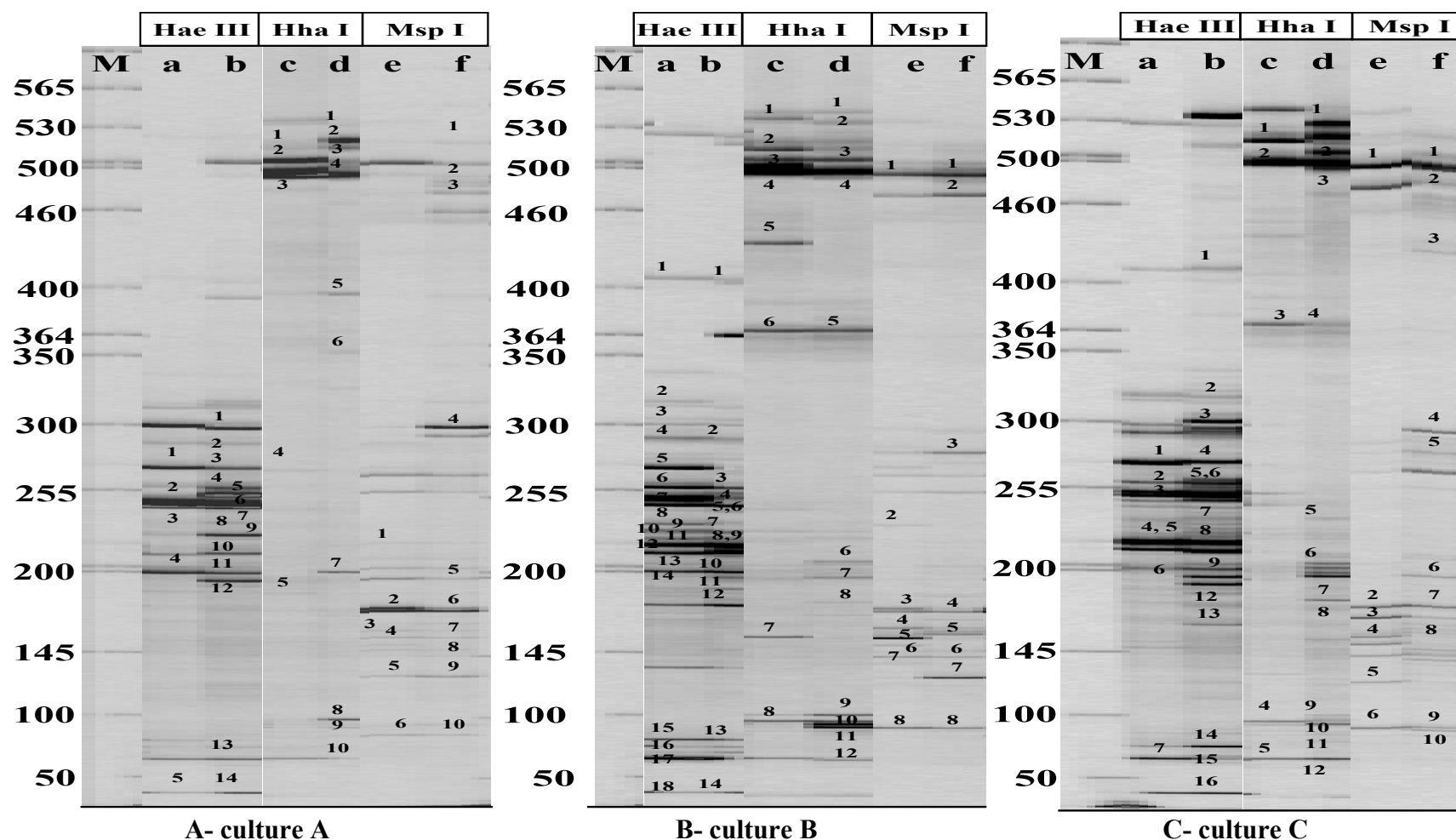
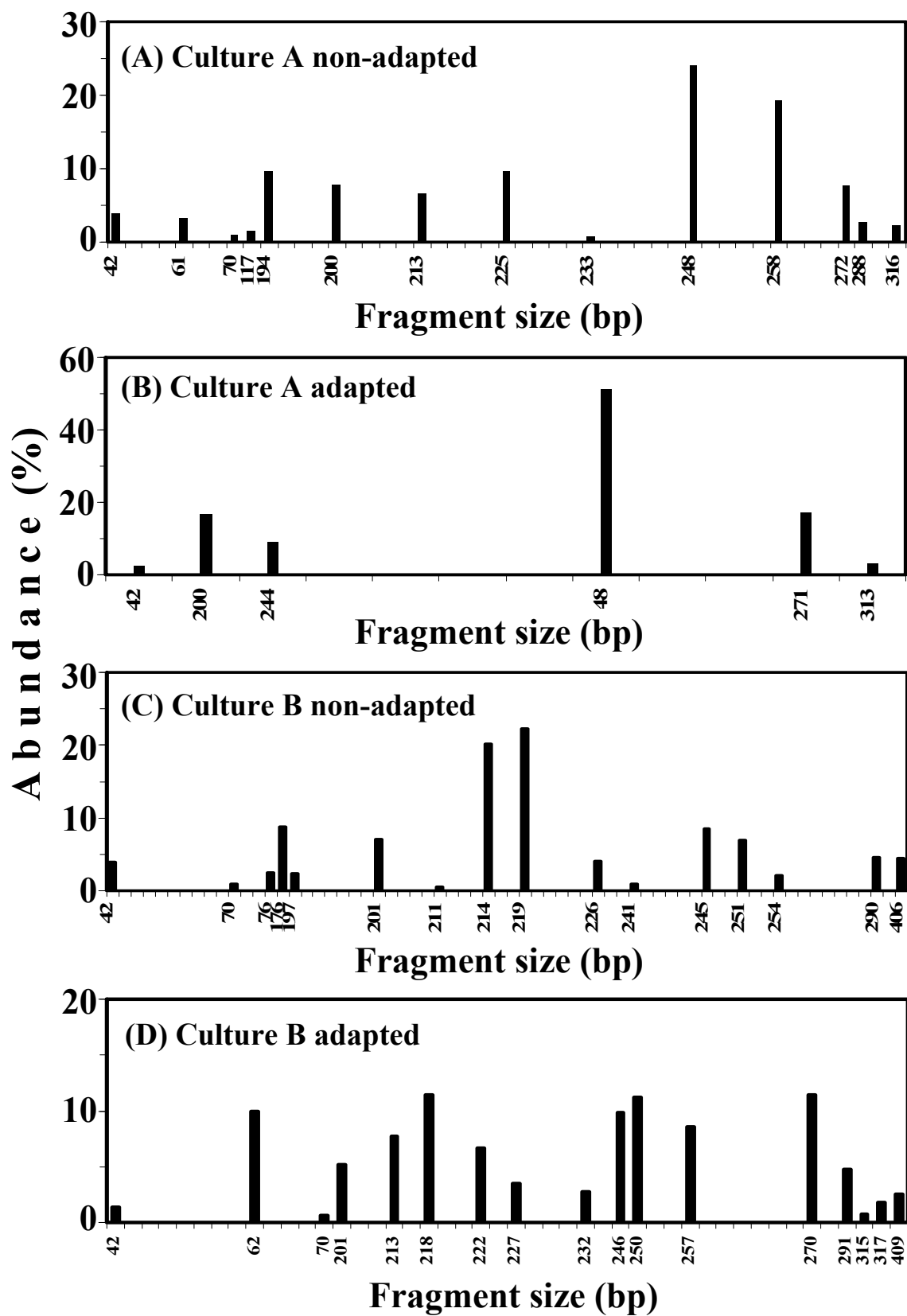


Figure 5.5 Bacterial community profiles as a function of glucose adaptation in: (A) culture A, (B) culture B, and (C) culture C as detected by terminal restriction fragment length polymorphism of PCR-amplified 16S rRNA gene fragments. Lanes a, c, and e are the samples after adaptation to glucose, and lanes b, d, and f are the samples before adaptation. Numbers shown on each lane indicate bands identified.



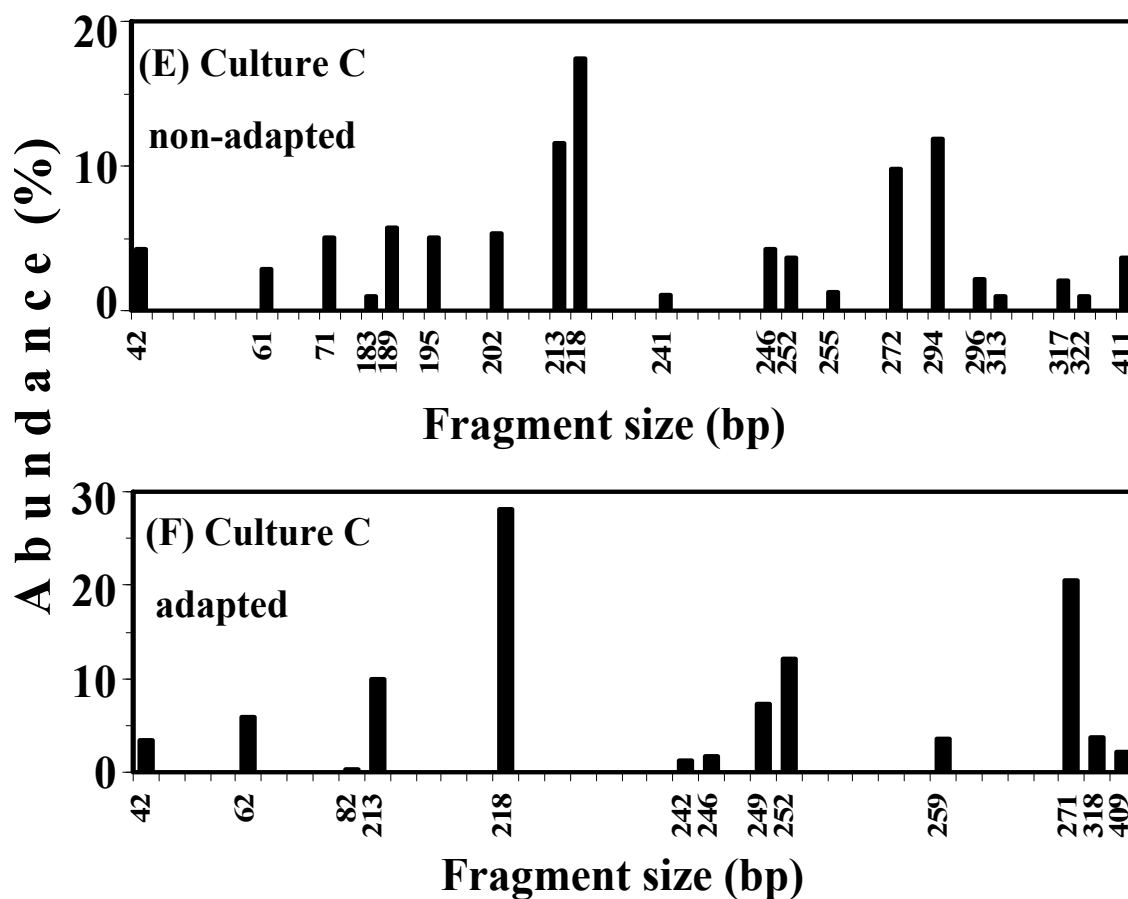


Figure 5.6 Comparison of (A) culture A non-adapted, (B) culture A adapted (C) culture B non-adapted, (D) culture B adapted, (E) culture C non-adapted and (F) culture C adapted based on the terminal fragments detected for the enzyme *Hae* III.

Table 5.6 Shannon diversity index for culture A, B, and C before and after 12 months of adaptation to glucose (mean  $\pm$  standard deviation for triplicate samples).

<u>Culture</u>	<u>Shannon Diversity index</u>	
	<u>adapted</u>	<u>non-adapted</u>
A	1.94 $\pm$ 0.14	3.32 $\pm$ 0.03
B	3.54 $\pm$ 0.04	3.64 $\pm$ 0.08
C	2.88 $\pm$ 0.04	3.21 $\pm$ 0.17

adaptation to glucose [lane a in Figure 5.5 (A) and Figure 5.6 (B)], culture A was dominated by *Mycoplasma* and *Victivallis vandensis* (band 2) 51.11%, followed by *Capnocytophaga* (band 4) 16.94%; *Clostridia* (band 1) 17.72%; *Eubacteria*, *Bacillus*, and *Desulfovibrio* (band 3) 9.03%.

Culture B was dominated by, before adaptation to glucose [lane b in Figure 5.5 (B) and Figure 5.6 (C)], *Geobacter Acidimicrbium*, *Bervibacillus*, and *pelobacter* (band 12 ) 22%; *Geobacter sulfurreducens* and *Therombaculum* (bands 9 and 8) 20%; *Bacillus* (bands 3 and 6) 8.5%; *Fusobacterium* (band 2) 4.58%; *Citrobacter*, *E. Coli*, *Erwinia*, and *Entrobacter* (band 14) 3.89%; *Desulfovibrio* (band 1 and 3) 2% while *Clostridia* (bands 10 and 11) formed 4%. H<sub>2</sub> oxidizing bacteria such as *Nocardia crassostrae* str. OB3 (band 17) and *Gordonia* (band 13) 1% were detected in culture B before adaptation to glucose. After adaptation to glucose, culture B [lane a in Figure 5.5 (B) and Figure 5.6 (D)] included *Clostridia* (band 18) 11%; *Gleobacter* (band 5) 11%; *Nocordia* and *Gordonia* (band 16) 10%; *Bacteroides*, *Flavobacterium*, *Actinobacillus*, and *deslufobacterium* (band 6) 9%; *Geobacter*, *Geobacillus*, *Pelobacter*, and *Eubacterium* (bands 12 and 13) 8%; *Citrobacter*, *E. Coli*, *Erwinia*, and *Entrobacter* (band 18) 1 %. Generally, glucose-adapted samples from culture B included fewer sulfate-reducing bacteria [bands 5, 7, and 14] than non-adapted samples [bands 3, 6, 9, 10, 11, and 13].

Before adaptation to glucose, Culture C [lane b in Figure 5.5 (C) and Figure 5.6 (E)] contained *Bervibacillus* (band) 17 %; *Rubrobacter* (band) 16%; *Clostridia* (band) 15%; *Cytopagha* (band) 4%; *Eubacteria* (band) 4 %; *Acinetobacter* and *Bifidobacteria* (band) 4%; *Bacteroides* and *Parabacteroides* (band) 3%. After adaptation to glucose, culture C [lane a in Figure 5.5 (C) and Figure 5.6 (F)] was dominated by *Geobacter*, *Geobacillus*

and *Thermobaculum* (band ) 28%; *Desulfobacter* (band) 21%; Eubacteria, *Pelobacter*, *Rubrobacter* (band) 10%; *Nocordia* and *Gordonia* (band ) 6%; *Clostridia* and *Actinobacillus* (band ) 4% *E. Coli*, *Erwinia*, *Cytophaga*, and *Entrobacter* (band) 3%.

Adaptation of culture C to glucose eliminated *Lactobacillus* [band 5 in non-adapted samples (lane b in Figure 5.5 (C))] and decreased *Clostridia*. The number of *Clostridia* bands [bands 4 and 5 in non-adapted samples] decreased after adaptation [band 1, (lane a in Figure 5.5 (C))]. *Bacteroides* [band 15 in non-adapted culture C samples] and *Parabacteroides* disappeared after adaptation to glucose. *Escherichia coli* appeared in glucose-adapted culture C samples [band 8].

In summary, *Brevibacillus* and *Syntrophobacter* species disappeared from all cultures after adaptation. Species that disappeared from at least two cultures after adaptation included *Bacteroides* (in cultures A and C), *Parabacteroides* (in cultures A and C), *Fusobacterium* (in cultures A and B), *Thermoanaerobacter* (in cultures A and C) (notice that some of these species were not detected in the third culture before adaptation). Other species that disappeared from at least one culture after adaptation include *Acidovora*, *Butyrivibrios* (in culture A), *Flavobacterium* (in culture A), *Pseudetharailcus* (in culture A), *Arthrobacter*, *Blastopirellua*, *Cyclobacter*, *Dehalobacter*, *Desulfobulbus*, *Desulfovibrio*, *Desulfouromonas*, *Geobacter*, *Halibacter*, *Paracoccus*, and *Victivallius* (in culture B), *Acinetobacter*, *Cytophaga*, *Dethiosulfovibrio*, *Gleobacter*, *Paenibaccillus* (in culture C).

*Clostridia* species such as *C. asparagiforme*, *C. quercicolum*, *C. asparagiforme*, *C. quercicolum*, *C. ramosum*, and *C. rectum* disappeared from at least one culture after



glucose-adaptation. Note that *C. botulinum* dominated in two cultures (B and C) and *C. Clostridiiforme* in one culture (A) after adaptation.

Species that were not affected by glucose-adaptation included *Bacillus*, *Citrobacter*, *Entrobacter*, *Eubacterium*, *Rubrobacter*, *Pelobacter*, *Gordonia*, *E. coli*, and *Falvobacterium* (in two cultures). Species that were detected only after glucose-adaptation in at least two cultures (B and C) included *Entrobacter*, *Erwinia*, *E. coli*, *Eubacterium*, and *Geobacillus* whereas *Nocordia*, *Neisseria*, and *Thermoanaerobacter* were detected in just one culture (B) after glucose-adaptation. *Thermoanaerobacter* disappeared from culture A and C but dominated in culture B after glucose-adaptation. *Falvobacterium* also disappeared from culture A, but was unaffected by glucose-adaptation in cultures B and C.

### **5.3.5 Microbiological data analysis using PCA**

PCA was used to compare cultures A, B, and C before and after 12 months of adaptation to glucose using the results of the T-RFLP analysis (Figure 5.7). The input for the analysis included the species' identification by the molecular weight (bp) and abundance as represented by the relative area. PCA was conducted for each enzyme (*Hae* III, *Hha* I, and *Msp* I) individually; scores were calculated using the T-RFLP profiles as the data sets for the three enzymes. Two principal components (PCs) explained 88% of the total variability in the *Hae* III data set, two PCs explained 56% of the variance in the *Hha* I data set, and two PCs explained 55% of the variance in the *Msp* I data set. PCA score maps showed that for each of the enzymes there were distinct differences

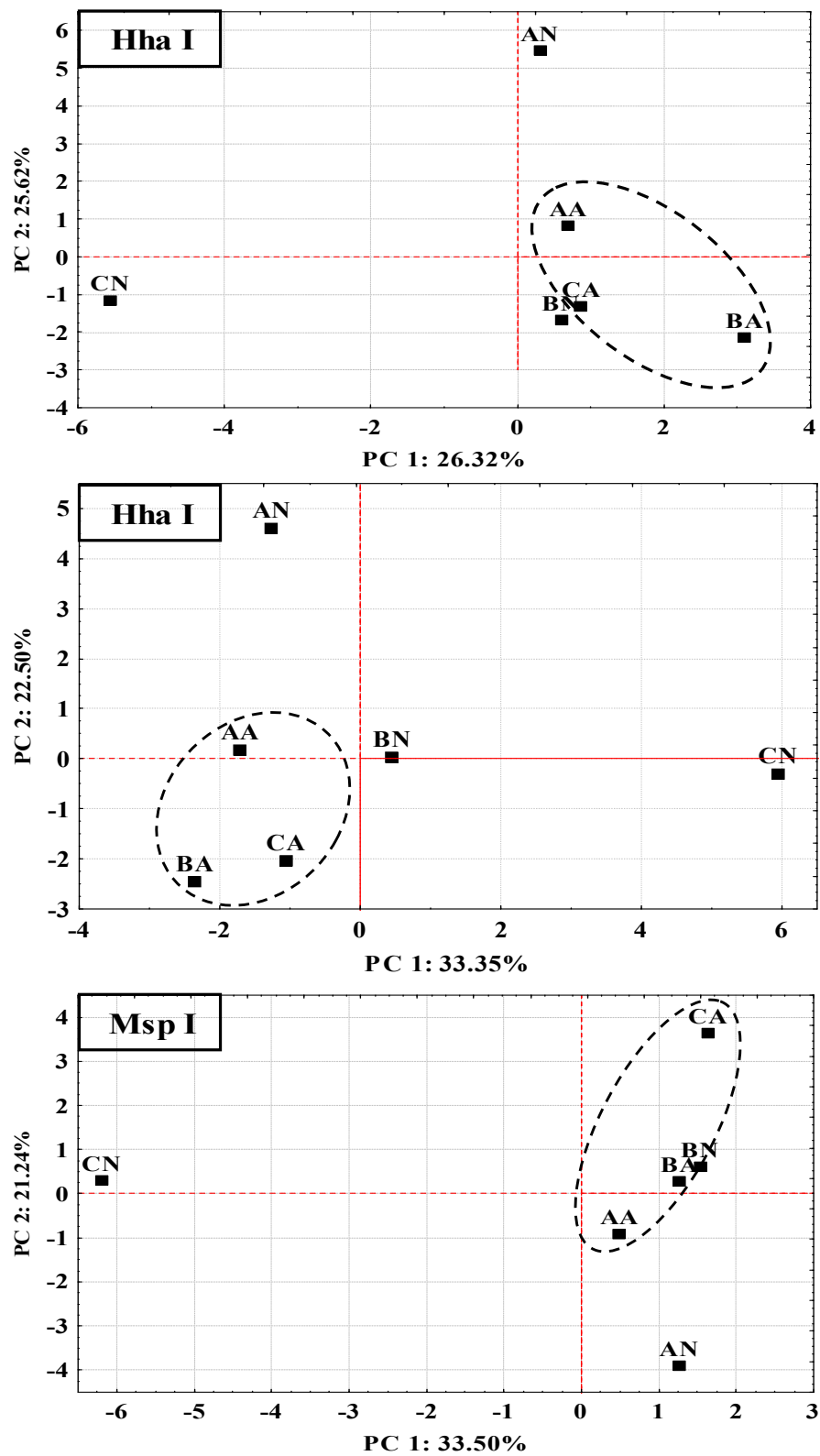


Figure 5.7 PCA of the data set of molecular weights for bacterial bands from T-RFLP gel image. (AA, BA, and CA = culture A, B, and C after 12 month of adaptation to glucose. AN, BN, and CN = culture A, B, and C before adaptation to glucose).

between the samples from cultures A, B, and C before and after glucose-adaptation. Note the enclosed clustering of the glucose-adapted samples in comparison to the scattered non-adapted samples (Figure 5.7).

## 5.4 DISCUSSION

Mixed culture dark fermentation can be classified as a "black box" process and the understanding of its microbial consortia suffers from a lack of phylogenetic and metabolic data on the dominant microorganisms. Although adaptation may be very important in H<sub>2</sub> production (Abreu *et al.*, 2009), no work investigating the evolution of inoculum in response to acclimatization processes and the effect on H<sub>2</sub> yield has been reported. Little information is available regarding changes in the structure and activity of the culture after long-term adaptation. In this study, the effects of long-term adaptation of three anaerobic cultures from different sources to glucose were monitored using a combination of kinetic and molecular genetic methods. Long-term adaptation to glucose had a positive impact on H<sub>2</sub> yield and the acidogenic trophic group in the mixed cultures; increased the yield by approximately 7%. The various trophic groups of microorganisms present in the initial inoculums showed different patterns of population change.

Culture A contained higher percentages of glucose-, acetate-, propionate-, and H<sub>2</sub>-degraders compared to cultures B and C. These results are in agreement with the findings of Moreno-Andrade and Bultron (2004) that the brewery culture designated as A contained more individual trophic groups per gram VSS than the municipal culture (C) UASB culture. A comparison of cultures A and C reveals that there is not much difference between them in terms of the total active biomass both before and after adaptation (Table 5.1). The results of the biomass concentration agrees with the findings

of Von Munch *et al.* (1999) in that acidogens and acetogens formed 70 to 90% of a mixed culture while methanogens formed 10%. Moreover, the results are consistent with the findings of Mackie and Bryant (1981) who reported the percentages of some trophic groups in mixed anaerobic culture as follows: acetate (3 %), propionate (0.06%), butyrate (0.4%), and H<sub>2</sub> (15 to 26%).

Lactate maximum specific utilization rates for all three cultures were relatively higher than the corresponding rates for other substrates likely because of the high fermentative activity of these cultures. This might be explained by comparing fermentative versus syntrophic degrading potentials. Although lactate is degraded syntrophically through an electron transfer processes, it could be fermented as well. Species with fermentative metabolism such as *Pelobacter propionicus* and *Desulfobulbus propionicus* ferment lactate to acetate and propionate (Schink 1984; Stams *et al.* 1984; Samain *et al.* 1982). Moreover, homoacetogens could ferment lactate solely to acetate (Braun *et al.* 1981; Andreesen *et al.* 1970). The higher maximum specific growth rates of fermentative bacteria in comparison with syntrophs (Stams, 1994), and the experimental observation that acetate was the only liquid by-product detected in lactate degradation assays support the explanation that the higher maximum specific lactate utilization rates are attributable to fermentative activity.

Acidogenic bacteria ferment glucose to propionate, butyrate, ethanol, and lactate and simultaneously releasing large amounts of free energy per electron. Thus, these microorganisms could support rapid growth. The observed increase in the proportion of the active acidogenic trophic group within the biomass after 12 months of adaptation to glucose is in agreement with previously reported data showing that adaptation to new

substrate selects for microorganisms with high specific substrate affinity (Temudo *et al.*, 2008). Such adaptation relates to the microbial ability to acquire or to activate enzymes for substrates not previously available to the organism (Kabler, 1996); thus, the kinetic properties of a cell may change due to adaptation (Kovarova-Kovar and Egli, 1998). These previously reported observations explain the increase in glucose-degraders' activity seen in this study after 12 months of glucose-adaptation (Table 5.1). Genetic changes cannot be excluded because mutational changes identified during adaptation to glucose-limitation in 280 generations of *E. coli* were in cellular components that constitute the high-affinity glucose transport pathway (Ferenci, 1996).

Statistical comparison of kinetic data did not find differences between glucose-adapted and non-adapted cultures. This is likely because of the existence of metabolic redundancy in culture composition, confirmed by the microbiological analysis. The activity of particular species may change with environmental conditions, but in general, the culture will maintain sufficient substrate-consuming activity. The increase in acidogenic activity is favourable to increasing the H<sub>2</sub> production and higher yields. This is consistent with previously reported adaptation-induced changes that include elevated levels of transporters and enzymes, changes in the cell surface, and metabolic changes leads to increased acid production (Padan *et al.*, 2005). The H<sub>2</sub> yields from glucose-adapted and non-adapted cultures were significantly different. PCA of the H<sub>2</sub> yield data set also indicated that glucose-adapted and non-adapted cultures are different and confirmed the effect of glucose-adaptation over long periods. Moreover, PCA of T-RFLP's profiles confirmed the differences due to adaptation because score plots revealed differences between the microbial communities.

The measured maximum specific substrate utilization rates for the various trophic groups in cultures A, B, and C lie within the range of previously reported data (Table 2.8). However, such comparisons should be performed very carefully because many past studies have used mixed cultures which were refrigerated prior to use in laboratory studies (Shizas *et al.*, 2001). The anaerobic metabolic activity of stored anaerobic mixed cultures is affected by length of storage, storage temperature, and exposure to air. The length of the storage period is reported to affect the metabolic activities of different microbial trophic groups; in particular, it can affect butyrate-, acetate-, and propionate-degraders. According to Wu *et al.* (1995), during eighteen months of storage at 22°C the degradation rates of butyrate, acetate and propionate decreased by 80, 70, and 50 %, respectively. Conklin *et al.* (2008) reported that the maximum specific substrate activities of a mixed culture decreased by 11 to 17% after refrigerated storage and concluded that refrigerated culture storage should be avoided. Cold storage, used to reduce the die-off rate of microorganisms, affected the activities of acetate-, propionate- and butyrate-degraders; it decreased the degradation rates for the three acids gradually according to Wu *et al.* (1995). Storage of granules at 4°C for one month decreased the butyrate-degradation rate by 45%. The maximum methanogenic-activity of thermophilic (55°C) granular culture decreased by 50% after storage at 30°C for 50 days. For example, the decay coefficients were higher at higher storage temperatures. The decay coefficients at lower temperatures range between 0.01 to 0.027 d<sup>-1</sup> for short-chain VFA-degraders in anaerobic cultures and between 0.004 to 0.037 d<sup>-1</sup> for acetate-degraders (Pavlostathis and Giraldo-Gomez, 1991). The history of culture maintenance should be considered when comparing metabolic activities of different cultures because the kinetic properties of a

cell may change due to adaptation (Kovarova-Kovar and Egli, 1998). In comparison with the data reported in Table 2.8, the measured rates in this work reflected the effects of the environmental and operational conditions, maintenance, feeding pattern, and growth medium. This is consistent with results reported by Dolfing and Bloemen (1985) that the activities of the biomass after three months of cultivation reflected the composition of the substrate.

Microbiological analysis revealed changes in the composition of the cultures due to glucose-adaptation, and supported previous findings of molecular 16S RNA studies which indicated changes in the composition of complex anaerobic microbial communities over time (Hallenbeck and Ghosh, 2009). T-RFLP profiles revealed some specific phylotypes for each culture besides the common phylotypes which appeared in all cultures. *Bacteroides*, *Clostridia*, *Eubacteria*, and *Lactobacillus* were dominant in an anaerobic digester (Toerien and Hattingh, 1969). *Clostridia*, *Bacillus*, *Entrobacter*, *Fusobacterium*, and *Klebsiella* are H<sub>2</sub>-producing acidogens that are common in anaerobic digestion (Britz *et al.*, 1994). All these species are capable of fermenting glucose and produce different quantities of H<sub>2</sub> and VFAs. Disappearance of *Bacteroides*, *Parabacteroides*, and *Lactobacillus* after glucose-adaptation may have contributed to the increased H<sub>2</sub> production seen in glucose-adapted cultures. The presence of species with very small areas (percentages) in T-RFLP output profiles may indicate their inactive metabolic reactions; however, their DNA will still be detected in low concentrations (Wagner *et al.*, 1995). Comparing the T-RF profiles of the bacterial populations using PCA demonstrated that adaptation to glucose created different bacterial communities.

The maximum degradation rate of each substrate by mixed cultures depends on the concentration and activity of the particular trophic group that catabolizes the substrate (Aguiar *et al.*, 1995). Differences in the maximum specific substrate utilization rates of different cultures under the same environmental conditions represent differences in the species of bacterial populations, or their growth conditions. The disappearance of species such as *Fusobacterium* and *Syntrophobacter* (e.g., *Syntrophus aciditrophicus*) from cultures A, B, and C may explain the decrease in the maximum specific utilization rates of butyrate and propionate. *Syntrophus aciditrophicus* (strain SB) is a strict anaerobic, Gram-negative, non-spore-forming bacterium that degrades certain fatty acids (saturated and unsaturated) to acetate, CO<sub>2</sub>, H<sub>2</sub>, and possibly formate in syntrophic association, but does not do so with H<sub>2</sub>/formate-using methanogen microorganisms or with sulfate-reducers (Swiss Institute of Bioinformatics, 2010). Syntrophic acetogens grow slowly even under optimum conditions with a minimum doubling time of 1.5 to 4 days (Mosey, 1983); their doubling time is 2.3 days with butyrate (Gujer and Zehnder, 1983) and 4.6 to 5.8 days with propionate (Boone and Bryant, 1980). Moreover, H<sub>2</sub> production from propionate and longer fatty acids is favorable only under low concentrations of H<sub>2</sub> (Schink, 1997). Therefore, the metabolism of these compounds by H<sub>2</sub>-producing acetogenic bacteria depends on the activity of H<sub>2</sub>-consuming methanogens. Notice the association between the decrease in the maximum specific utilization rate of H<sub>2</sub> and those of propionate, butyrate, and lactate. This supports previous findings that the rate of VFA-degradation is proportional to H<sub>2</sub>-consuming activity (Voolapalli and Stuckey, 1999).

Hydrogen-consuming bacteria were detected in cultures A, B, and C. *Nocardia* and *Paracoccus* are H<sub>2</sub>-oxidizing bacteria (Gottschalk, 1986). *Desulfovibrio* and *Desulfobacter*



are sulfate-reducing bacteria (SRB) that contain hydrogenase and are able to use  $H_2$  as an energy source if a suitable carbon source exists (Sokatch 1969; Levett, 1990). In the absence of sulfate, *Desulfovibrio* sp. can grow syntrophically with hydrogenotrophic methanogens (Bryant *et al.*, 1977). In addition, they are capable of fermentative metabolism. Lactate is the preferred substrate for SRBs such as *Desulfovibrio* (Sokatch 1969; Gottschalk, 1986). The reduction in the dominance of *Desulfovibrio* and *Desulfobacter* after glucose-adaptation might partially account for the decrease in  $H_2$ -consumption activity in all cultures after adaptation to glucose. SRB species such as *Desulfovibrio* declined, but were not eliminated; likely because of their fast fermentative growth with pyruvate and fumarate in comparison to that of *Syntrophobacter* species (Kuever *et al.*, 1986). Furthermore, *Syntrophobacters* species are unlikely to dominate a culture because of their slow growth rates with VFAs such as propionate (Kuever *et al.*, 1986).

Redundancy in the metabolic functions of bacteria in mixed cultures makes it difficult to correlate specific activities to the population (or percentage of the biomass) of the specific trophic group. In anaerobic fermentative systems, microorganisms may have similar growth rates and yields and many can catalyze several shared fermentative reactions at the same time (Rodriguez *et al.*, 2006). For example, *Butyrivibrio* and *Fusobacterium* are butyrate-forming bacteria (Gottschalk, 1986), *E. Coli*, *Erwinia* and *Entrobacter* ferment sugars to lactate, acetate, formate, ethanol,  $H_2$ , and  $CO_2$ . However, *Erwinia* and *Entrobacter* produce less acid, but more  $CO_2$  and ethanol (Gottschalk, 1986). *Clostridium*, *Fusobacterium*, *Bacteroides*, and *Klebsiella* can produce  $H_2$ ; however, *Bacteroides* and *Klebsiella* are not high  $H_2$ -producing bacteria (Liu *et al.*, 2008).

compared to *Clostridia*. Facultative anaerobic bacteria such as *Eubacterium*, *Entrobacter*, and *Klebsiella* either were not affected or became dominant after glucose-adaptation (Kumar and Das, 2001). Although their higher abundances do not necessarily imply higher physiological activities than those of the strict anaerobes, it would be surprising if these high numbers of facultative anaerobes do not contribute significantly towards the total physiological activities under the incubation conditions. Surprisingly, Oh *et al.* (2003) found that 15 aerobic species such as *Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp. from a suspended mixed culture produced between 1.02-1.22 mol H<sub>2</sub>·mol<sup>-1</sup> glucose under anaerobic conditions. *Lactobacillus* ferment sugars to lactic acid with a small quantity of acetate (Sokatch, 1969; Kandler and Weiss, 1986). *Firmicutes* are VFA- degrading syntrophic bacteria (Riviere *et al.*, 2009). *E. coli* and *Bacillus* use the Embden-Meyerhof pathway to ferment glucose (Sokatch, 1969). Until now, there were no tools or methods available to quantify concurrent metabolic activities of the various species in a mixed culture sample.

The acclimatization times required to obtain methanogen-free acidogenic inocula generating H<sub>2</sub> and organic acids are between 3 to 30 days according to Valdez-Vazquez and Poggi-Varaldo (2009). However, experimental evidence indicates that the microbial population is still changing even after 60 days of adaptation; the changes include intermediate enzyme activities and the concentration of microorganisms (Hattingh *et al.*, 1967). Therefore, more long-term adaptation studies are required to assess the effects of adaptation on culture activity under different stress conditions. The length of the adaptation course in this study (12-months) ensures confidence in the results, as there was enough time for the microbial population to stabilize. Microbial adaptation to a substrate

has been attributed to: (i) mutation or genetic exchanges; (ii) selective enrichment of a minor population capable of degrading the substrate; (iii) enzyme regulation; and (iv) DNA rearrangements (Becker *et al.*, 2006; Barkay and Olson, 1986). However, the mechanism of microbial community adaptation is not well understood and further research is required in order to predict when adaptive events occur.

Disappearance of some bacteria from one culture and their dominance in another after adaptation might reflect the existence or absence of microbial interactions among that species and other microorganisms in that specific culture. These interactions could enable the dominance of a microorganism; otherwise, that species would disappear from the culture. *Flavobacterium sp.* is an example; in culture A, it disappeared after glucose-adaptation whereas in cultures B and C it was unaffected. Likewise, *Victivallis Vadensis* disappeared from culture B, but was unaffected in culture A. *Victivallis Vadensis* grows optimally at 37 °C with a doubling time of 0.5 h in a pH range of 5 to 7.5; it converts glucose to acetate, ethanol, and H<sub>2</sub> in pure culture or, with a methanogenic partner, syntrophically to acetate and H<sub>2</sub> (Zoetendal *et al.*, 2003). *Victivallis Vadensis* shares with some fermentative organisms the capability to grow on extracellular polymers and dead cells (Wu *et al.*, 1995). Likewise, *Cytophaga*, a genus of fermentative microorganism, degrades bio-macromolecules (Cottrell and Kirchman, 2000).

The combination of kinetic, microbiological, and principal components analyses explains the effects of long-term adaptation to glucose on the three cultures. The kinetic approach and the determination of individual metabolic activities could be used to monitor and detect potential unbalanced situations among the different bacterial species in H<sub>2</sub> production processes.

## 5.5 CONCLUSIONS

Long-term acclimatization to glucose improved the yield of  $H_2$  up to  $3.32 \pm 0.12$  mol  $H_2 \cdot mol^{-1}$  glucose through decreasing the biomass concentration and activity of the  $H_2$ -consuming population. Although LA increased the  $H_2$  yield in both glucose-adapted and glucose-non-adapted cultures, it significantly minimized  $H_2$ -consumption in glucose-adapted cultures.

Different cultures showed different percentages of biomass concentration and activities of the various active trophic groups both before and after adaptation to glucose. Generally, the percentage of the total active biomass increased after adaptation, mainly due to an increase in the concentration of glucose-degraders, however, the concentrations of other trophic groups decreased. Adaptation to glucose affects the activities of lactate-, propionate-, and butyrate-degraders. *Clostridia*, *Geobacter*, *Bacillus*, *Bacteroides* are the main species detected in the three mixed cultures characterized. However, the dominating species after acclimatization are not the same in the different cultures. Adaptation changed the microbial community profiles; some species disappeared completely while others became dominant after the adaptation to glucose. PCA of the metabolic by-products,  $H_2$  yields, and microbiological T-RFLP's profiles indicated differences between glucose-adapted and glucose-non-adapted cultures; moreover, PCA demonstrated similarity among glucose-adapted and LA-treated cultures.

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## **CHAPTER 6: EFFECTS OF LINOLEIC ACID AND pH 5.0 ON THE KINETICS OF A BIOHYDROGEN-PRODUCING ANAEROBIC MIXED CULTURE**

### **6.1 INTRODUCTION**

Hydrogen (H<sub>2</sub>) production during the dark fermentation of carbohydrates is the focus of potential commercial-scale applications. In terms of H<sub>2</sub> yield, mixed culture inocula with or without pre-treatment, are as effective as pure obligate anaerobes such as *Clostridia*, *Bacillus*, *Escherichia coli*, and *Citrobacter* (Table 2.4 and 2.5). Using mixed cultures are advantageous over pure culture because they are cheap and can be obtained from wastewater treatment facilities. Moreover, mixed culture can utilize non-sterilized substrate including low value organic wastes.

The H<sub>2</sub> yield from mixed culture depends on the structure and the individual activities of the microbial community present in the reactor, the operational and environmental parameters, the type and concentration of substrates, and the presence of toxic compounds (McHugh *et al.*, 2003). These factors affect the performance, the activity, and ultimately the fate of the microbial community (Demirel and Scherer, 2008). Fermentative, acidogenic, acetogenic, and homoacetogenic bacteria, apart from hydrogenotrophic and aceticlastic methanogens (Demirel and Scherer, 2008), are the major trophic groups of the mixed anaerobic cultures. Hence, understanding the kinetics of these trophic groups in mixed cultures is essential to developing H<sub>2</sub>-producing systems to their fullest potential. Such understanding is complicated by dynamic factors; in particular, the complex nature of the mixed microbial populations and their metabolic interactions (Strydom *et al.*, 2001).

The activity and performance of the major trophic groups within the anaerobic H<sub>2</sub>-producing consortia are still not well defined. Researchers have focused on fermenters and acidogens, but not on homoacetogens and acetogens (Dolfing, 1988; Schink, 1994; Abreu *et al.*, 2007). Relatively little is known about the functional importance of homoacetogenic metabolism in anaerobic digestion. Similarly, published studies covering the behavior, performance, activities, and fate of homoacetogens in fermentative H<sub>2</sub> production are scarce in the scientific literature (Demirel and Scherer, 2008). In fermentation, electron equivalents in glucose can be directed to as many pathways which are thermodynamically favorable. Therefore, defining the limiting reaction rate in H<sub>2</sub> production requires quantifying the biomass composition in terms of the individual trophic groups and their corresponding activities under the optimum stress conditions. An acceptable and precise measure for the quantification of the microorganisms in the H<sub>2</sub> producing consortium is still an open research area requiring more experimental work (Ruggeri *et al.*, 2009).

In mixed culture fermentation, pH influences the performance of the various trophic groups in the consortia because of their different optimum pH ranges. Low pH of 5.0-5.5 was reported to be optimum for H<sub>2</sub> production (Li and Fang, 2007). However, maintaining low pH alone to maximize H<sub>2</sub> yield is limited by the eventual presence of acetogenic and methanogenic H<sub>2</sub>-consuming microorganisms. An increasing number of studies reported low pH methanogenesis (Jain and Mattiasson, 1998; Brauer *et al.*, 2006; Taconi *et al.*, 2007; Castello *et al.*, 2009). Therefore, keeping the pH low is not sufficient to sustain methane-free H<sub>2</sub> production or to prevent H<sub>2</sub> consumption by homoacetogens.

A number of pretreatments have been examined and are not sufficient on their own to increase the H<sub>2</sub> yield from mixed anaerobic cultures; these include microwave, ultrasonication, chemical supplementation, chemical inhibition using BESA and iodopropane, acid treatment, base treatment, freeze-thawing, and thermal treatment. Although most pretreatment methods are able to minimize methane (CH<sub>4</sub>) production no work to date have shown these methods are able to produce H<sub>2</sub> yield over sustainable periods. For example, heat pretreatment alone was either not sufficient or was inconsistent in developing a suitable microbial consortium for H<sub>2</sub> production (Kotay and Das, 2009). Surprisingly, heat pre-treatment could not completely remove methanogens, which partially resumed growth under favorable conditions (Oh *et al.*, 2003; Wang *et al.*, 2007).

Long-chain fatty acids (LCFAs) have shown to improve the H<sub>2</sub> yield in comparison with other pretreatment methods. LCFAs are natural biodegradable organic polymers which can be obtained as a waste stream from food-processing plants. LCFAs inhibit several bacteria involved in the anaerobic digestion process (Koster and Cramer, 1987; Sayed *et al.*, 1988; Thiele *et al.*, 1988; Lalman and Bagley, 2002), and impair syntrophic interactions between microbial groups (Tay and Yan, 1996). Hanaki *et al.* (1981) concluded that LCFAs affect obligate H<sub>2</sub>-producing acetogenic bacteria, hydrogenotrophic and acetotrophic methanogenic archaea that convert the intermediates resulting from the  $\beta$ -oxidation process to methane (Miranda *et al.*, 2005).

It is important to know how individual trophic groups from H<sub>2</sub>-producing consortia would be affected by different stresses imposed on the anaerobic consortia (e.g. altered pH, heat treatment, and chemical inhibition). Species resistant to an inhibitor will be

selected during long-term exposure, and CH<sub>4</sub> formation could return to the levels observed before the inhibitor was introduced (Ungerfeld *et al.*, 2004).

The objectives of this study are: first, to quantify the effects of the presence of LA as a chemical inhibitor and keeping pH low (5.0) on the activity and proportions of the various trophic groups in H<sub>2</sub>-producing anaerobic mixed culture at 37°C; and second, to examine the effect of LA on the diversity of the microbial community using the molecular genetic method of T-RFLP.

## **6.2 MATERIALS AND METHODS**

Materials and methods for this research are fully described in chapter 3. The source of the inoculum used and the maintenance protocol are detailed in section 3.1. Preparation of all serum batch reactors followed the protocol described in section 3.2. The kinetic characterization procedure is detailed in section 3.3 and the equations used in the calculations are given in Appendix C. The experimental design matrix is described in section 3.4.2. Analytical methods for analyzing gas by-products (H<sub>2</sub>, CH<sub>4</sub> and CO<sub>2</sub>), and liquid by-products (VFAs and alcohols) are described in section 3.5.

Experiments for kinetic studies and H<sub>2</sub> production were conducted in four stages. Kinetic experiments (stages I, II, and IV) determined the percentage and maximum specific utilization rates of active degraders of glucose, lactate, acetate, propionate, butyrate, and H<sub>2</sub> at pH 7.6, pH 5.0, and pH 5.0 plus 2,000 mg L<sup>-1</sup> linoleic acid (LA). Stage III was a H<sub>2</sub> production study.

## 6.3 RESULTS

### 6.3.1 Kinetic data

The preparation of the batch reactors in each stage was as follows: In Stage I: six sets of triplicate batch reactors with an initial pH 7.6. In Stage II: six sets of triplicate batch reactors with an initial pH 5.0. In Stage III: two sets of triplicate batch reactors with an initial pH 5.0; the first set was fed 5,000 mg L<sup>-1</sup> glucose whereas the second set was fed 5,000 mg L<sup>-1</sup> glucose plus 2,000 mg L<sup>-1</sup> LA. In Stage IV: six sets of triplicate batch reactors with an initial pH 5.0 were prepared and fed 2,000 mg L<sup>-1</sup> LA. All experiments were carried out at a temperature of 37±2°C. Results of the kinetic study [percentage of biomass concentration of an active trophic group ( $X_a$ ) and the corresponding maximum specific substrate utilization rate ( $k_a$ )] are shown in Table 6.1.

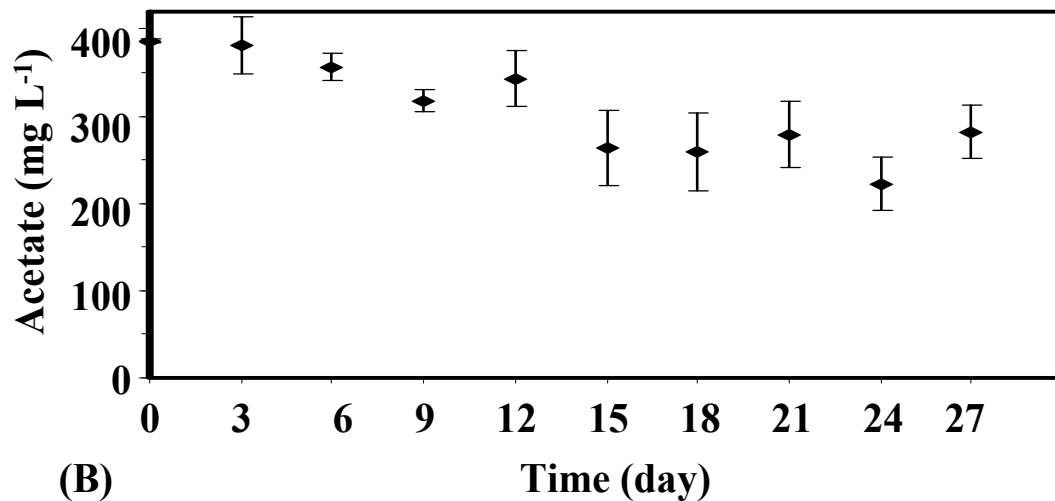
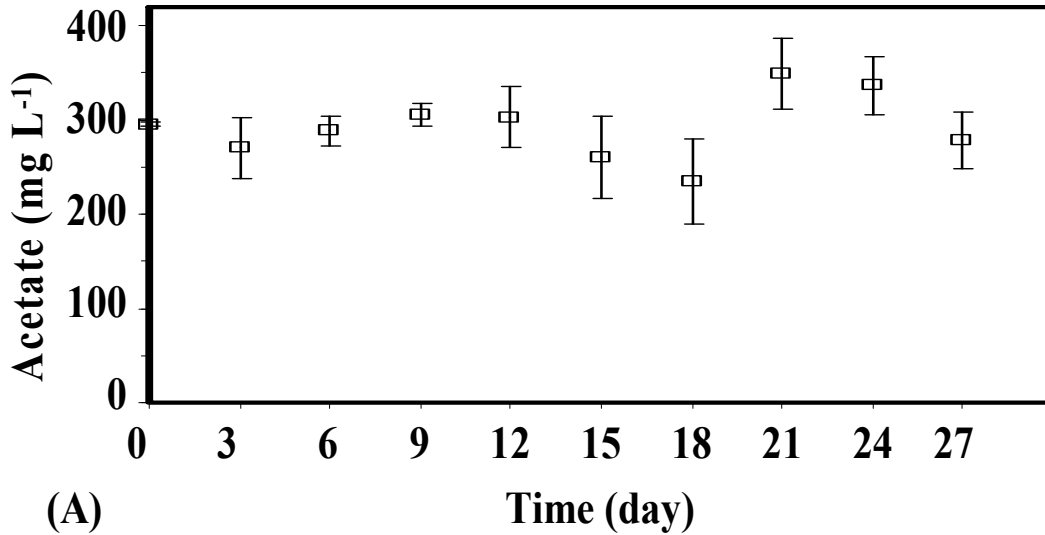
Substrate degradation line curves used to calculate the maximum substrate utilization rate and the corresponding percentage of active biomass are shown in Appendix C. The maximum substrate utilization rate and the corresponding percentage of active biomass were not determined in phase III.

Acetate, butyrate, or propionate (100 mg L<sup>-1</sup>) was added to culture treated with 2,000 mg L<sup>-1</sup> LA and incubated at initial pH 5.0 (Figure 6.1). For the culture incubated with acetate and LA, it was not possible to differentiate the acetate resulting from LA degradation from that injected; the acetate profiles in the LA-control and acetate plus LA-fed bottles did not correlate (Figure 6.1A and B). Generally, butyrate degraded very slowly after a lag phase of approximately 5 days.

Table 6.1 Percent biomass concentration of active trophic groups and maximum specific substrate utilization rates for cultures of sets I, II, and IV.

Substrate	$k_a$ (d <sup>-1</sup> )			$X_a$ (%)		
	Set I pH 7.6	Set II pH 5.0	Set IV pH 5.0 + LA	Set I pH 7.6	Set II pH 5.0	Set IV pH 5.0 + LA
Glucose	10.40±1.25 <sup>a</sup>	35.98±8.62 <sup>b</sup>	26.42±1.73 <sup>c</sup>	11.19±2.31 <sup>d</sup>	4.14±1.03 <sup>e</sup>	3.47±1.68 <sup>f</sup>
Lactate	31.13±5.83 <sup>a</sup>	64.05±15.57 <sup>b</sup>	2.25±0.11 <sup>c</sup>	1.56±0.71 <sup>d</sup>	0.73±0.25 <sup>d</sup>	0.06±0.01 <sup>e</sup>
Acetate	1.65±0.78 <sup>a</sup>	1.40±0.18 <sup>a</sup>	--	6.91±1.87 <sup>d</sup>	3.91±0.19 <sup>d</sup>	--
Butyrate	1.90±0.35 <sup>a</sup>	2.26±0.68 <sup>a</sup>	--	2.31±0.76	1.45±0.29 <sup>d</sup>	--
Propionate	3.16±0.68 <sup>a</sup>	4.47±0.82 <sup>a</sup>	--	0.89±0.28 <sup>d</sup>	0.77±0.04 <sup>d</sup>	--
Hydrogen	20.56±0.97 <sup>a</sup>	15.62±2.27 <sup>a</sup>	1.08±0.21 <sup>b</sup>	4.15±1.25 <sup>d</sup>	0.99±0.04 <sup>c</sup>	1.70±0.06 <sup>f</sup>
Total active biomass				27.01±3.93 <sup>a</sup>	11.99±1.71 <sup>b</sup>	--

Note: Superscripts <sup>a, b</sup> and <sup>c</sup> indicate significantly different means for  $k_a$  in the same row. Superscripts <sup>d, e</sup>, and <sup>f</sup> indicate significantly different means for  $X_a$  in the same row. No comparisons should be made between rows.



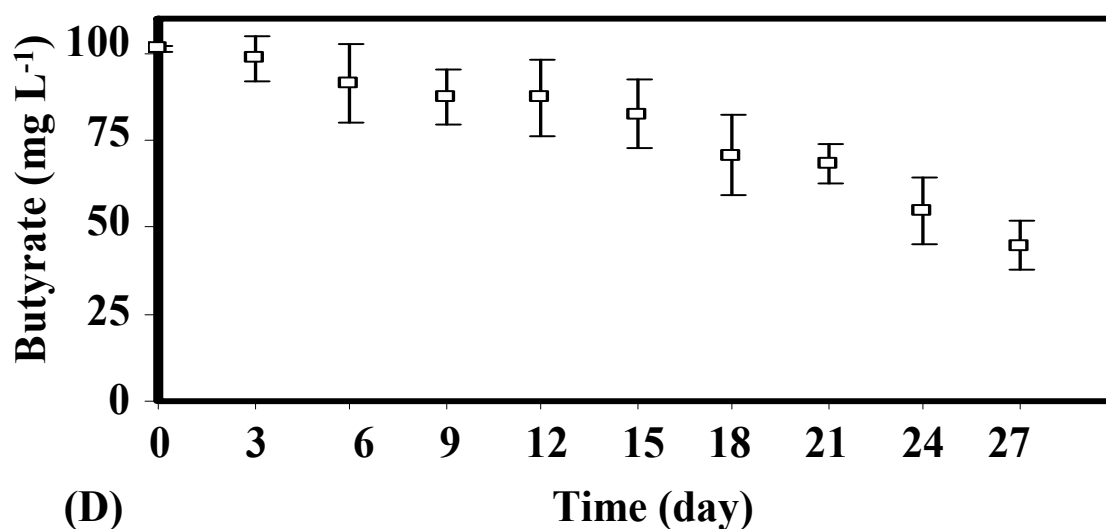
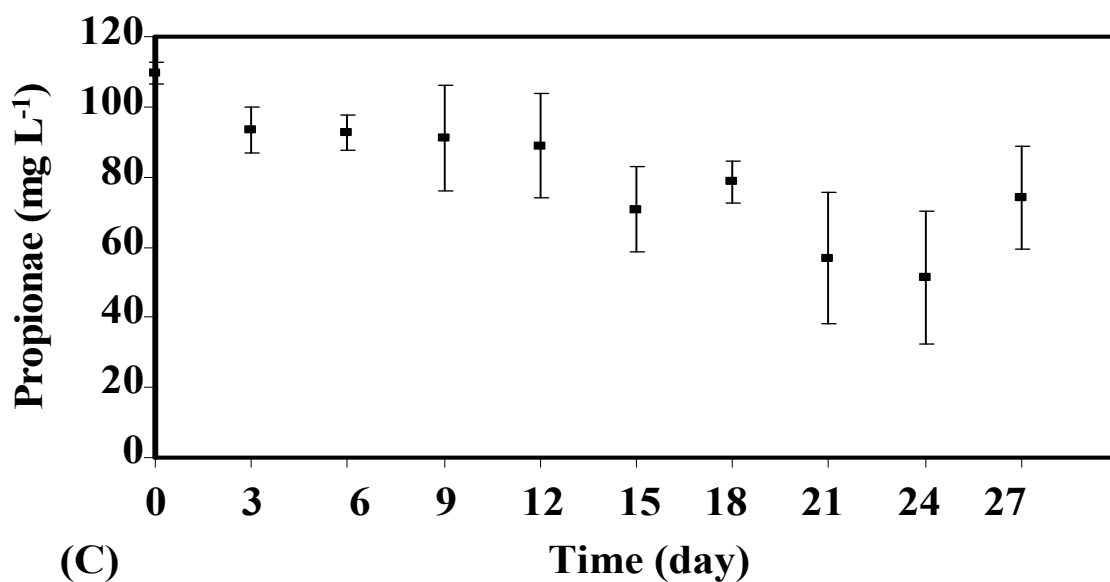


Figure 6.1 Profiles of (A) acetate production in culture fed 2,000 mg L<sup>-1</sup> LA (LA-control), (B) acetate in culture fed 100 mg L<sup>-1</sup> acetate and 2,000 mg L<sup>-1</sup> LA, (C) butyrate in culture fed 100 mg L<sup>-1</sup> propionate and 2,000 mg L<sup>-1</sup> LA, and (D) butyrate in culture fed 100 mg L<sup>-1</sup> propionate and 2,000 mg L<sup>-1</sup> LA. (Means  $\pm$  standard deviation for triplicate samples are shown).

The propionate degradation profile (Figure 6.1C) was flatter than that of butyrate (Figure 6.1D); some increases in the propionate concentration were observed on Days 15, 24, and 27. It was not possible to apply the kinetic protocol to estimate both  $k_a$  and  $X_a$ ; fitting the data from the degradation curve enabled an approximate value for only  $k_a$  to be



estimated. The specific substrate utilization rates were:  $0.002 \pm 0.006$  (acetate),  $0.001 \pm 0.0004$  (butyrate), and  $0.0009 \pm 0.0003$  (propionate)  $\text{mg COD L}^{-1} \cdot \text{mg} \cdot \text{VSS}^{-1} \cdot \text{d}^{-1}$ .

Activities of all trophic groups (except activities related to acetate and  $\text{H}_2$  utilization) increased whereas the percentage of active biomass concentration of all trophic groups decreased when the pH decreased from 7.6 to 5.0. The extent of changes in the maximum specific substrate utilization rates and the percentage of active biomass concentration are shown in Table 6.2 as a percentage relative to those measured at pH 7.6.

Table 6.2 Change in the maximum specific substrate utilization rate ( $k_a$ ) and percentage of active biomass concentration ( $X_a$ ) due to lowering the pH from 7.6 to 5.0 expressed as percent relative to those measured at pH 7.6.

<b>The change due to lowering pH from 7.6 to 5.0 expressed as percent relative to pH 7.6</b>		
<b><u>substrate</u></b>	<b><u><math>k_a</math></u></b>	<b><u><math>X_a</math></u></b>
<b>Glucose</b>	245.0	-63.00
<b>Lactate</b>	105.7	-53.21
<b>Acetate</b>	-15.15	-43.42
<b>Butyrate</b>	18.94	-37.23
<b>Propionate</b>	41.45	-13.48
<b>Hydrogen</b>	-24.03	-76.14

Post hoc analysis (pairwise comparisons: two-tailed t-tests at 95% confidence level) were used to determine the significance of differences between the means for the maximum specific substrate utilization rates of the samples of culture with an initial pH 7.6 (Set I) and samples with initial pH 5.0 (Set II). The pairwise comparisons revealed that the activity of glucose, lactate, acetate, and butyrate degrading microorganisms at pH 7.6 differs significantly from those in the same culture but with an initial pH 5.0 (Table 6.3). Similarly, the concentrations of active trophic groups of glucose, acetate, and  $\text{H}_2$  in the biomass are significantly different for the two initial pH values under consideration.

However, the activities of H<sub>2</sub> consumers were not significantly different for the two initial pH values under consideration.

Table 6.3 p-value for the statistical comparisons (two-tails t-test) of the means of the maximum specific substrate utilization rate ( $k_a$ ) and percent active biomass concentration ( $X_a$ ) for pH 7.6 and pH 5.0

<u>Substrate</u>	<b>p-value of two-tails t-test for comparisons of means of pH 7.6 and pH 5.0</b>	
	<u><math>k_a</math></u>	<u><math>X_a</math></u>
Glucose	<b>0.036</b>	<b>0.016</b>
Lactate	<b>0.041</b>	0.152
Acetate	<b>0.045</b>	<b>0.045</b>
Butyrate	<b>0.022</b>	0.152
Propionate	0.181	0.366
Hydrogen	0.193	<b>0.018</b>

Note: Numbers in bold font indicate significant difference between pH 7.6 and 5.0

The presence of LA clearly show a decrease in the activity of the various trophic groups. Acidogens lost approximately 27% of their glucose degrading activity. The impact of LA on lactate degraders was very strong with a decrease in the maximum substrate utilization rate of lactate by 97% (from 64 to 2 (d<sup>-1</sup>)). Similarly, the percentage of the active lactate degraders in the biomass decreased by 92% (from 0.73% to 0.06%) in the presence of LA. The most important effect of LA was on the activity of H<sub>2</sub> consuming microorganisms, which decreased by 93%, although the percentage of active H<sub>2</sub> degraders in the biomass increased by 72% in LA-treated culture compared to pH 5.0 alone.

Acetate, butyrate and propionate degraders were also significantly affected such that it took almost a month to degrade between 50 to 60 mg L<sup>-1</sup> of each of butyrate and propionate.

### 6.3.2 Hydrogen consumption at pH 7.6, pH 5.0, and pH 5.0 plus LA

The kinetics of H<sub>2</sub> consumption were determined in batch reactor experiments at pH 7.6, 5.0, and pH 5.0 plus LA. Lowering the pH from 7.6 to 5.0 decreased the maximum specific H<sub>2</sub> utilization rate by 24% (relative to the rate at pH 7.6). LA (2,000 g L<sup>-1</sup>) decreased the maximum specific H<sub>2</sub> utilization rate by 93% (relative to the rate at pH 5.0 without LA). This means the reduction is 99% relative to pH 7.6. Hence, lowering the pH reduced the rate by only 24% while LA caused 75% of the reduction in the maximum specific H<sub>2</sub> utilization rate. Pairwise comparisons (Tukey's multiple comparisons test at 95% confidence interval) revealed that the maximum H<sub>2</sub> specific utilization rates at pH 7.6 and pH 5.0 were not similar, and differed significantly from the rate for culture at pH 5.0 plus LA (Table 6.4).

Table 6.4 Hydrogen consumption rates in mixed anaerobic culture at pH 7.6, 5.0, and pH 5.0 plus LA at 37°C.

<u>Culture</u>	<u>pH</u>	<u>LA</u> <u>(mg L<sup>-1</sup>)</u>	<u>H<sub>2</sub> consumption rate</u> <u>(day<sup>-1</sup>)</u>
B	7.6	0	20.56±0.97 <sup>a</sup>
B	5.0	0	15.62±2.27 <sup>b</sup>
B	5.0	2,000	1.08±0.21 <sup>c</sup>

Note: means with similar superscripts are statistically similar.  
Means with different superscripts are significantly different.

### 6.3.3 Hydrogen production study

The H<sub>2</sub> production experiment was conducted in control and LA-treated cultures with an initial pH set at 5.0 concurrently with the kinetic studies. The yield of H<sub>2</sub> in glucose control and LA-treated samples are given in Table 6.5. The maximum H<sub>2</sub> yield of 3.19±0.09 mol H<sub>2</sub>·mol<sup>-1</sup> glucose was observed in LA-treated samples two days after the second glucose injection (Day 6). The maximum H<sub>2</sub> yield in the glucose control

( $2.33 \pm 0.24 \text{ mol H}_2 \cdot \text{mol}^{-1} \text{ glucose}$ ) was also measured on Day 6. The maximum  $\text{H}_2$  yields after the first glucose injection in glucose control and glucose-fed plus LA-treated cultures were  $1.82 \pm 0.29$  and  $2.74 \pm 0.10 \text{ mol H}_2 \cdot \text{mol}^{-1} \text{ glucose}$  (Day 4). A two-tailed t-test at 95% confidence level was conducted to compare the  $\text{H}_2$  yield from control and LA-treated samples. Based on this test, the  $\text{H}_2$  yield in LA-treated samples was significantly different than the yield in the control samples after the first glucose injection (Days 2, 3, and 4), and after the second glucose injection (Days 6, 7, and 8).

Table 6.5 Hydrogen yield in stage III ( $\text{H}_2$  production experiment).

Incubation time (days)	<u><math>\text{H}_2</math> yield (<math>\text{mol H}_2 \cdot \text{mol}^{-1} \text{ glucose}</math>)</u>	
	<u>Control</u>	<u>2,000 (mg L<sup>-1</sup>) LA</u>
1	$0.37 \pm 0.37^a$	$1.05 \pm 0.05^a$
2	$0.68 \pm 0.13^a$	$2.07 \pm 0.05^b$
3	$1.65 \pm 0.36^a$	$2.48 \pm 0.17^b$
4	<b><math>1.82 \pm 0.29^a</math></b>	<b><math>2.74 \pm 0.10^b</math></b>
5	$1.67 \pm 0.72^a$	$2.46 \pm 0.11^a$
6	<b><math>2.33 \pm 0.24^a</math></b>	<b><math>3.19 \pm 0.09^b</math></b>
7	$1.94 \pm 0.25^a$	$2.99 \pm 0.01^b$
8	$1.60 \pm 0.56^a$	$2.92 \pm 0.36^b$

Notes:

1. Both cultures received  $5,000 \text{ mg L}^{-1}$  glucose on day 0 and day 4.
2. Superscripts <sup>a</sup> and <sup>b</sup> indicate statistically different means in the same row. No comparison should be made between different rows.
3. Bold font indicates maximum yield after the 1<sup>st</sup> or 2<sup>nd</sup> glucose injections

LA affected the acidogenic population by reducing the rate of glucose degradation (Figure 6.2). The glucose initial degradation rate decreased from  $2.60 \pm 0.10 \text{ } \mu\text{g glucose} \cdot \text{mg}^{-1} \text{ VSS} \cdot \text{min}^{-1}$  in the control samples to  $0.30 \pm 0.08 \text{ } \mu\text{g glucose} \cdot \text{mg}^{-1} \text{ VSS} \cdot \text{min}^{-1}$  in LA-treated samples. The mean glucose initial degradation rates in the control and LA fed samples were significantly different (Two tailed t-test;  $p \leq 0.05$ ).

Apparent  $H_2$  consumption was observed in glucose control and glucose-fed plus LA-treated samples after the second glucose injection. The observed  $H_2$  consumption rates in the triplicate samples of the glucose control culture were 0.83, 0.14, and 0.13  $\mu\text{mol}$

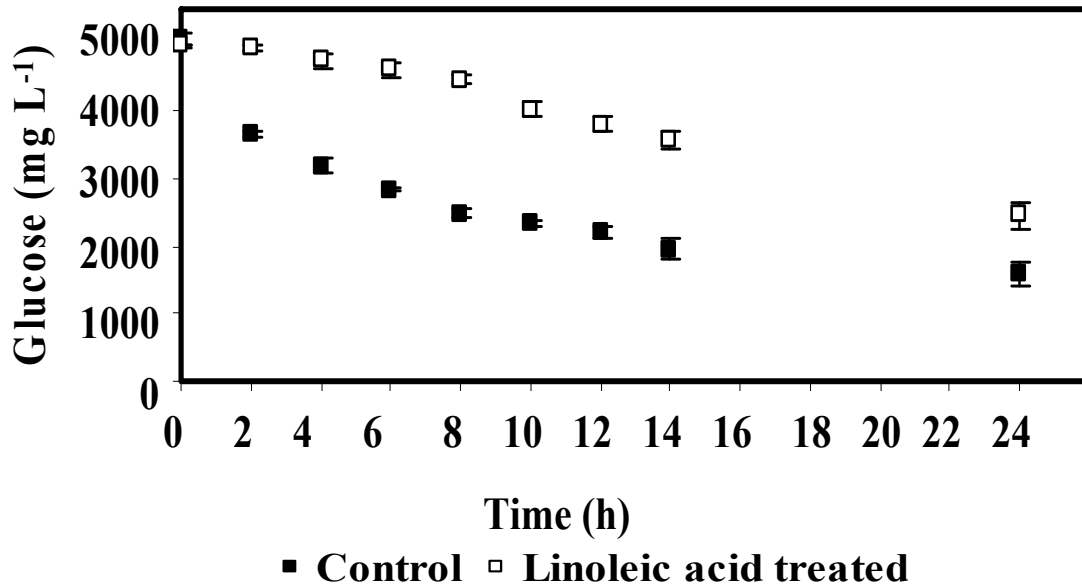


Figure 6.2 Glucose degradation profiles in cultures receiving 5,000  $\text{mg L}^{-1}$  plus 2,000  $\text{mg L}^{-1}$  LA. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

$H_2 \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$  while in the triplicate samples of the culture fed glucose and treated with LA were 0.67, 0.19, and 0.06  $\mu\text{mol } H_2 \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$ . The overall  $H_2$  consumption rates were  $0.36 \pm 0.40$  and  $0.31 \pm 0.32$   $\mu\text{mol } H_2 \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$  in glucose control and glucose-fed plus LA-treated cultures, respectively. Methane was produced after the first glucose injection in the glucose control and glucose-fed plus LA-treated samples to a maximum of  $514.2 \pm 7.95$  and  $3.88 \pm 0.96$   $\mu\text{mol CH}_4 \text{ bottle}^{-1}$ , respectively. This indicates that LA decreased  $\text{CH}_4$  production by more than 99%. No  $\text{CH}_4$  was observed in LA-treated samples after the second glucose injection.

### 6.3.4 VFAs and alcohols production during hydrogen production

The major VFAs produced were acetate, propionate, and butyrate (Figure 6.3A, B, and C). Control samples produced acetate to a maximum of  $853 \pm 53 \text{ mg L}^{-1}$  on Day 4 (four days after the first glucose injection), which increased by 20% (to  $1020 \pm 75 \text{ mg L}^{-1}$ ) on Day 8 (four days after the second glucose injection). Propionate was produced with a trend showing increasing accumulation in control samples ( $309 \pm 100$  and  $515 \pm 23 \text{ mg L}^{-1}$  on four days after the first and second glucose injections, respectively). Butyrate was produced in control samples to a maximum of  $362 \pm 53$  on Day 4 and  $390 \pm 9 \text{ mg L}^{-1}$  on Day 8 (four days after the first and second glucose injections, respectively).

LA-treated samples produced and accumulated increasing concentrations of acetate and butyrate. No propionate was detected in LA-treated samples. A maximum of  $1260 \pm 120$  and  $3020 \pm 50 \text{ mg L}^{-1}$  of acetate was measured in LA-treated samples on Days 4 and 8 (four days after the first and second glucose injections, respectively). Butyrate was produced in increasing concentrations and maximum levels of  $220 \pm 50$  (Day 4) and  $510 \pm 30 \text{ mg L}^{-1}$  (Day 8) were measured in LA-treated samples, four days after the first and second glucose injections, respectively.

Isopropanol, ethanol, and propanol were the major alcohols detected in the  $\text{H}_2$  production experiment (Figure 6.3D, E, and F). Control samples accumulated isopropanol and propanol up to a maximum of  $410 \pm 43$  and  $640 \pm 24 \text{ mg L}^{-1}$ , respectively, on Day 8 (four days after the second glucose injection). LA-treated samples accumulated propanol and ethanol up to maximum of  $430 \pm 25$  and  $270 \pm 16 \text{ mg L}^{-1}$ , respectively, on Day 8 (four days after the second glucose injection).

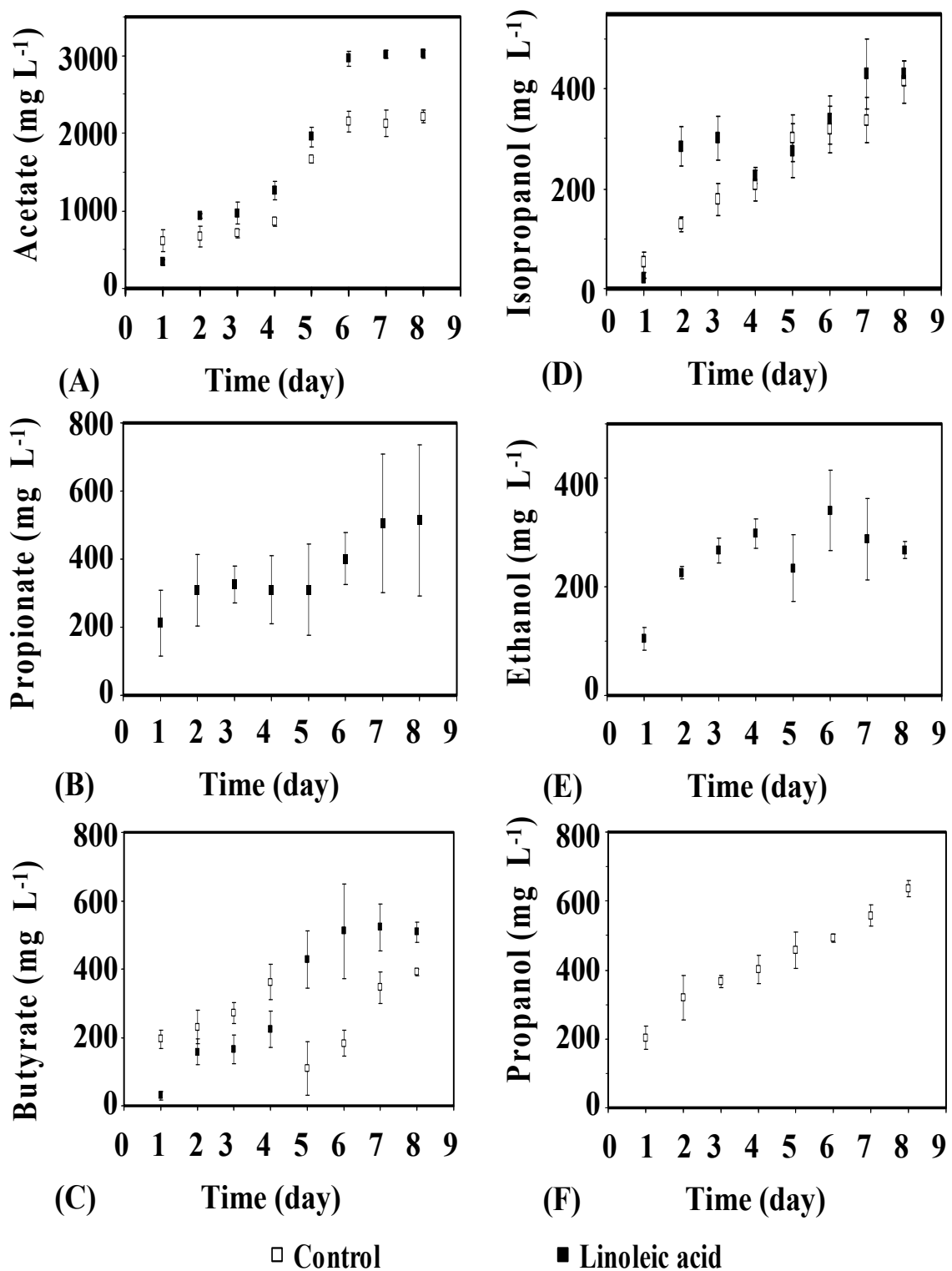


Figure 6.3 VFAs: (A) acetate, (B) propionate, and (C) butyrate; and alcohols (D) isopropanol, (E) ethanol, and (F) propanol production profiles in control and LA-treated culture (both received  $5,000 \text{ mg L}^{-1}$  glucose. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

### 6.3.5 Electron mass balance

The electron mass balance for control and LA-treated ( $2,000 \text{ mg L}^{-1}$ ) samples of the culture in  $\text{H}_2$  production experiments is presented in Figure 6.4. Electron recovery converged around 100% on Day 4 and on Day 6, four days after the first and two days after the second glucose injections (Figure 6.4A). Occasional recovery of greater than 100% of the electrons might have been due to possible biomass decay at the end of the fermentation. The electron mass balance data show that an increased percentage of electron equivalents were directed towards  $\text{H}_2$  in cultures treated with LA compared to controls (Figure 6.4B). Electron equivalents channeled to  $\text{H}_2$  in LA-treated samples after the second glucose injection accounted for 25% of the total electron equivalents available in glucose. In control samples,  $\text{H}_2$  accounted for only 17% of the total electron equivalents available in glucose after the second glucose injection.

Four days after the second glucose injection (Day 8), VFAs accounted for almost 35% of electron equivalents produced in control and 40% in LA-treated samples (Figure 6.4C). The LA-treated culture directed 10% more electron equivalents towards alcohol production than the control culture (Figure 6.4D); a maximum of 25% of the electron equivalents in glucose were synthesized as alcohols in control cultures, whereas in LA-treated cultures, it was only 18%.

The distribution of the electrons in the various by-products on specific days is shown in Figure 4.7. Propionate and acetate held most of the electron equivalents in control samples, whereas butyrate and acetate dominated LA-treated samples. LA affected the distribution of the electron equivalents and directed them towards  $\text{H}_2$  and butyrate instead of propionate.



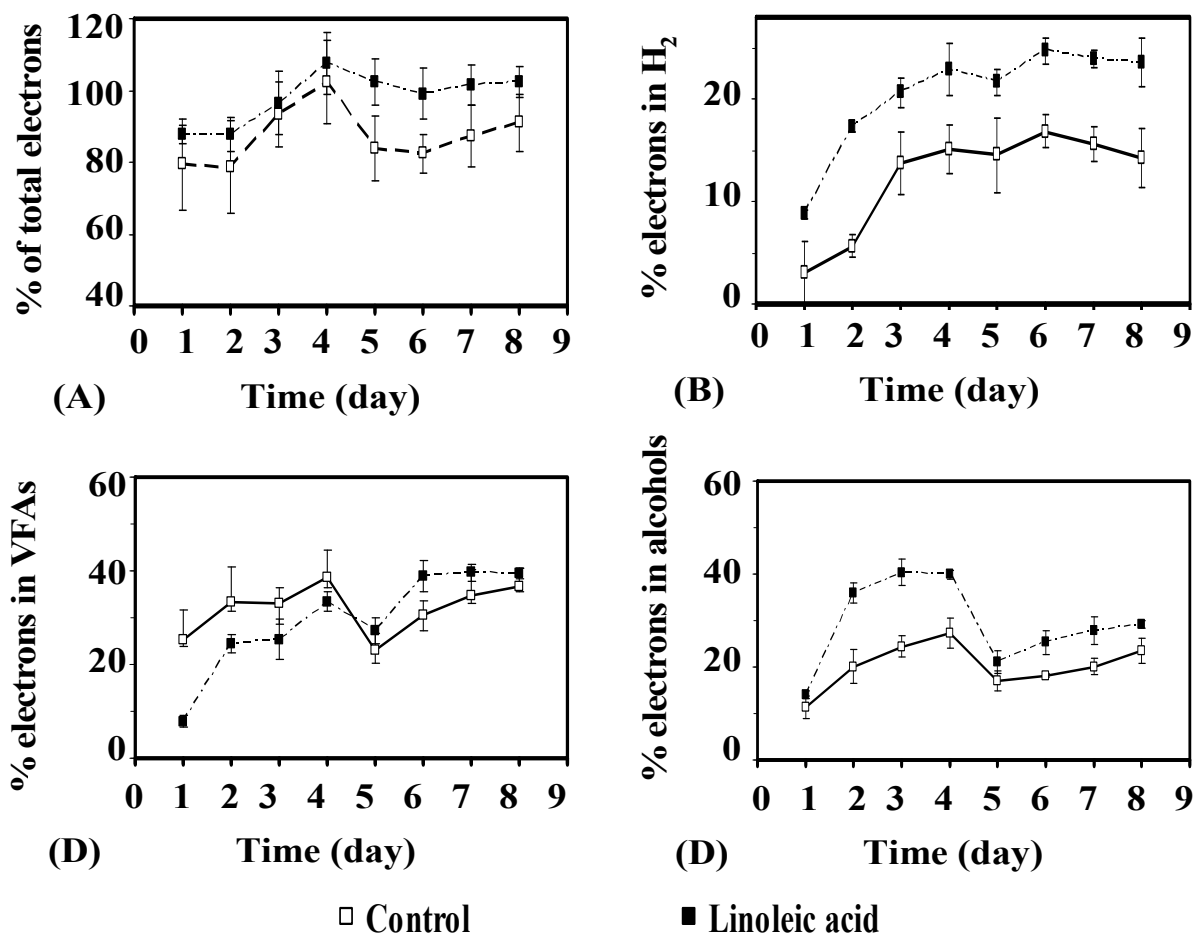


Figure 6.4 Electron mass balance for control and LA-treated samples in the hydrogen production experiment. All cultures were fed 5,000 mg L<sup>-1</sup> glucose. (Means  $\pm$  standard deviations for triplicate samples are shown).

### 6.3.6 Flux balance analysis (FBA) of H<sub>2</sub> data

MetaFluxNet software, Version.1.8.6.2 was used to perform the mass balance analysis of the metabolic fluxes in the glucose control and glucose-fed plus LA-treated cultures on a daily basis according to the metabolic reactions network of the model shown in Figure 3.3. The stoichiometries of the bioreactions (R1 to R30) are given in Table 3.9. The input included the external substrate (glucose) and the measured by-products (gases, VFAs, and alcohols). The output of the metabolic flux analysis on the day of the maximum H<sub>2</sub> yields

in control and LA-treated cultures is shown in Figure 6.5. The intercellular  $H_2$  yield predicted by FBA and measured intracellular  $H_2$  yields are shown in Figure 6.6. The quantity of  $H_2$  consumed by the homoacetogenic (R17) and solventogenic (R21) reactions are shown on the same figure to aid explanation of the scenario of  $H_2$  production over the entire course of fermentation.

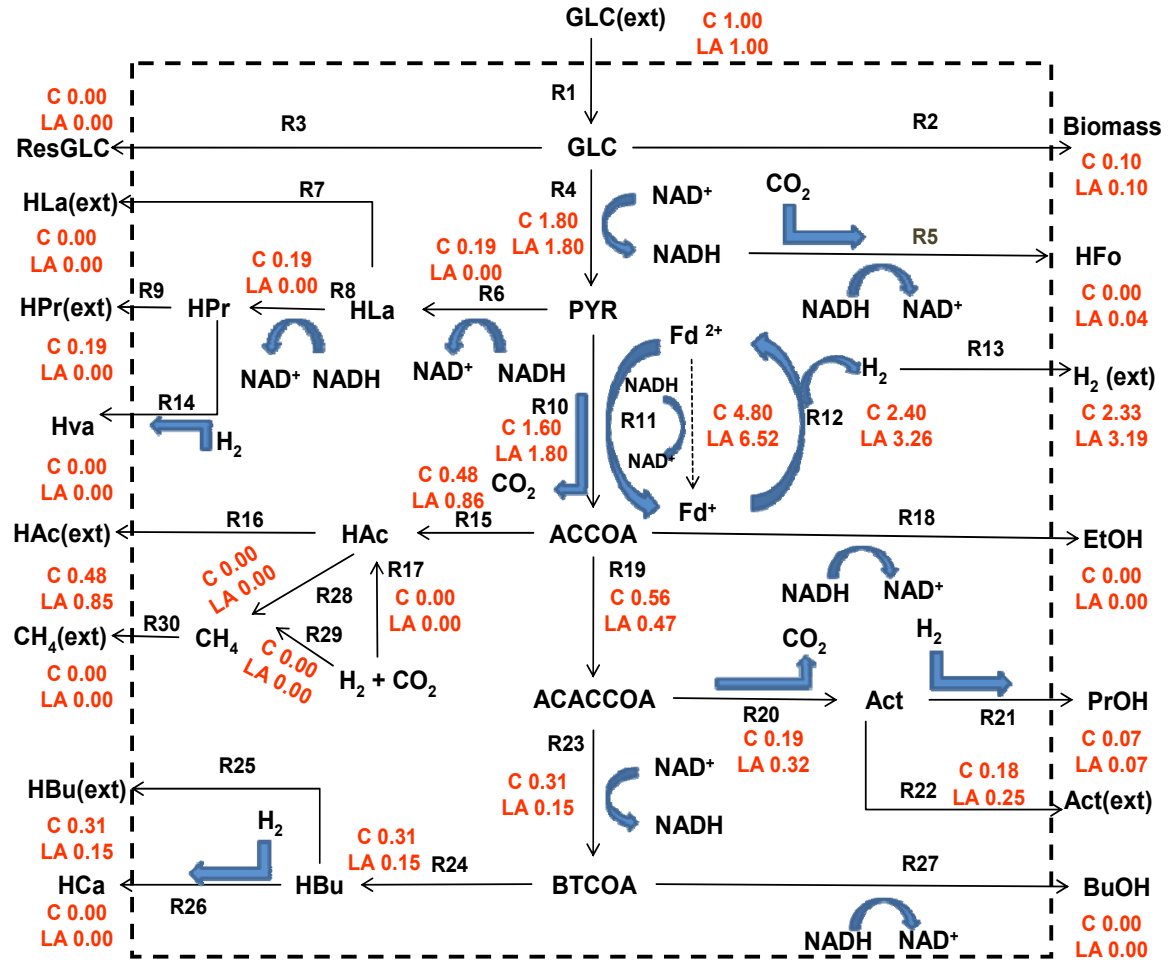
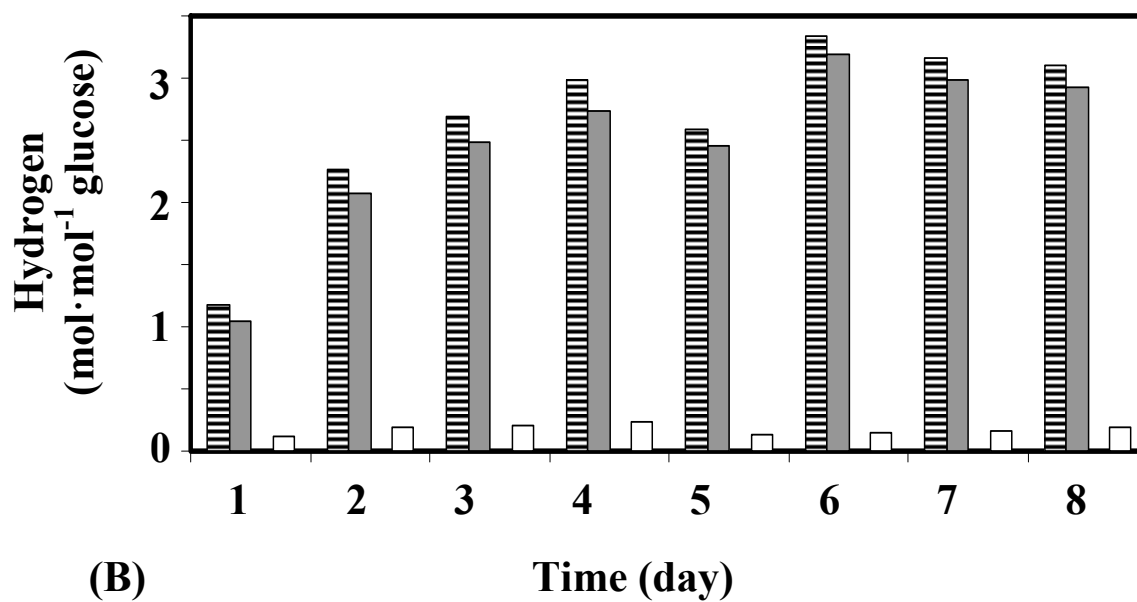
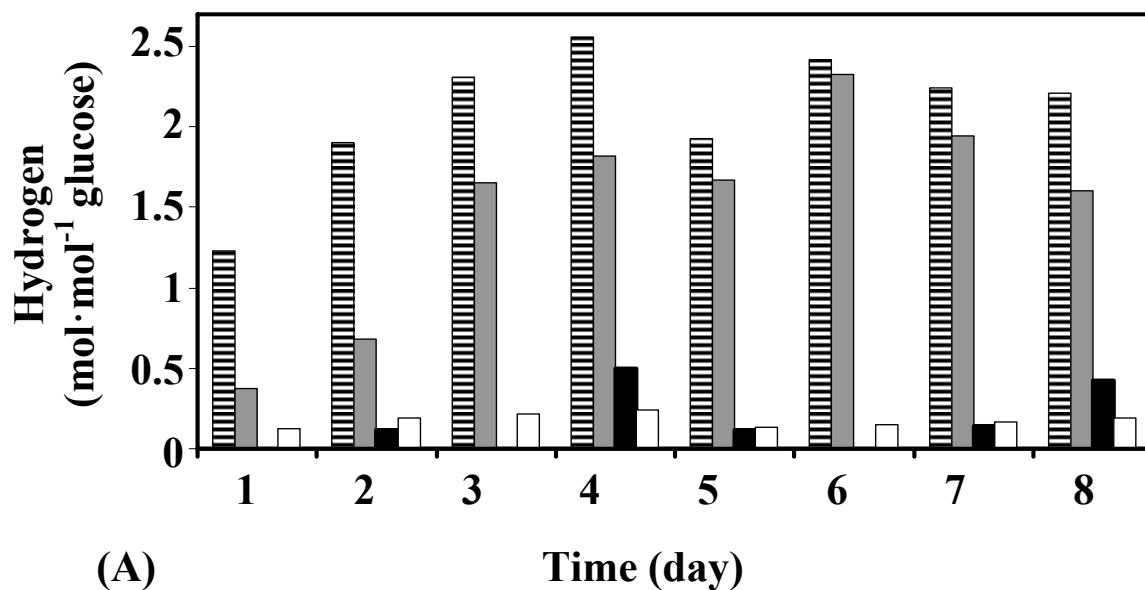


Figure 6.5 Flux balance analysis in control and LA-treated cultures for maximum  $H_2$  yield. (All samples fed 5,000 mg  $L^{-1}$  glucose).



■ Intracellular H<sub>2</sub> yield      ■ Extracellular H<sub>2</sub> yield  
 ■ Homoacetogenic H<sub>2</sub> consumption      □ Solventogenic H<sub>2</sub> consumption

Figure 6.6 Hydrogen yield and homoacetogenic and solventogenic H<sub>2</sub> consumption predicted by the model of the flux balance analysis for control and LA-treated culture. (All samples fed 5,000 mg L<sup>-1</sup> glucose).

FBA did not predict homoacetogenic H<sub>2</sub> consumption (R17) in the LA-treated culture after either the first or second glucose injections. Similarly, it did not predict

homoacetogenic H<sub>2</sub> consumption in the control culture after the first glucose injection; however, it predicted increasing homoacetogenic activity after the second glucose injection. On Days 7 and 8, the homoacetogens consumed 0.14 and 0.42 mol H<sub>2</sub> per mol of glucose injected. The solventogenic reaction (R21) consumed almost the same quantity of H<sub>2</sub> in the control and LA-treated cultures.

### **6.3.7 Microbiological characterization of hydrogen production and consumption experiments**

The molecular diversity of the bacterial community was investigated by nested-PCR of the 16S rRNA gene using the T-RFLP method. Samples of the culture under the different incubation conditions were analyzed to define the diversity and dominating species of microorganisms, and to detect the effects of LA on the culture composition. *Hae* III and *Hha* I T-RFLP profiles were obtained. The raw data sets were comprised of peaks reflecting the fragment size in base pairs (bp) of terminal restriction fragments (T-RFs) and the area of each peak measured in fluorescence units. The relative abundance of each band detected was determined by calculating the area of each peak as a percentage of the total area. The T-RFLP gel images are shown in Figure 6.7. The number of relatively abundant bacterial species, estimated by the mean richness determined from 16S rRNA T-RFLP *Hae* III profiles, are plotted as percentage (relative abundance) against fragment size (bp) in Figure 6.8.

The control samples from the H<sub>2</sub> production experiment (Lane a in Figure 6.7A) were dominated by *Bacteroides* sp. (band 6; 22.8%); *Bacillus* sp. (band 8; 19%); *Clostridium* purinolyticum (band 4; 9%); *Methyloarcula terricola* (band 13; 7%); *Acinetobacter calcoaceticus*, *Bacillus* sp., and *Falvobacterium* sp. (band 6; 6%);

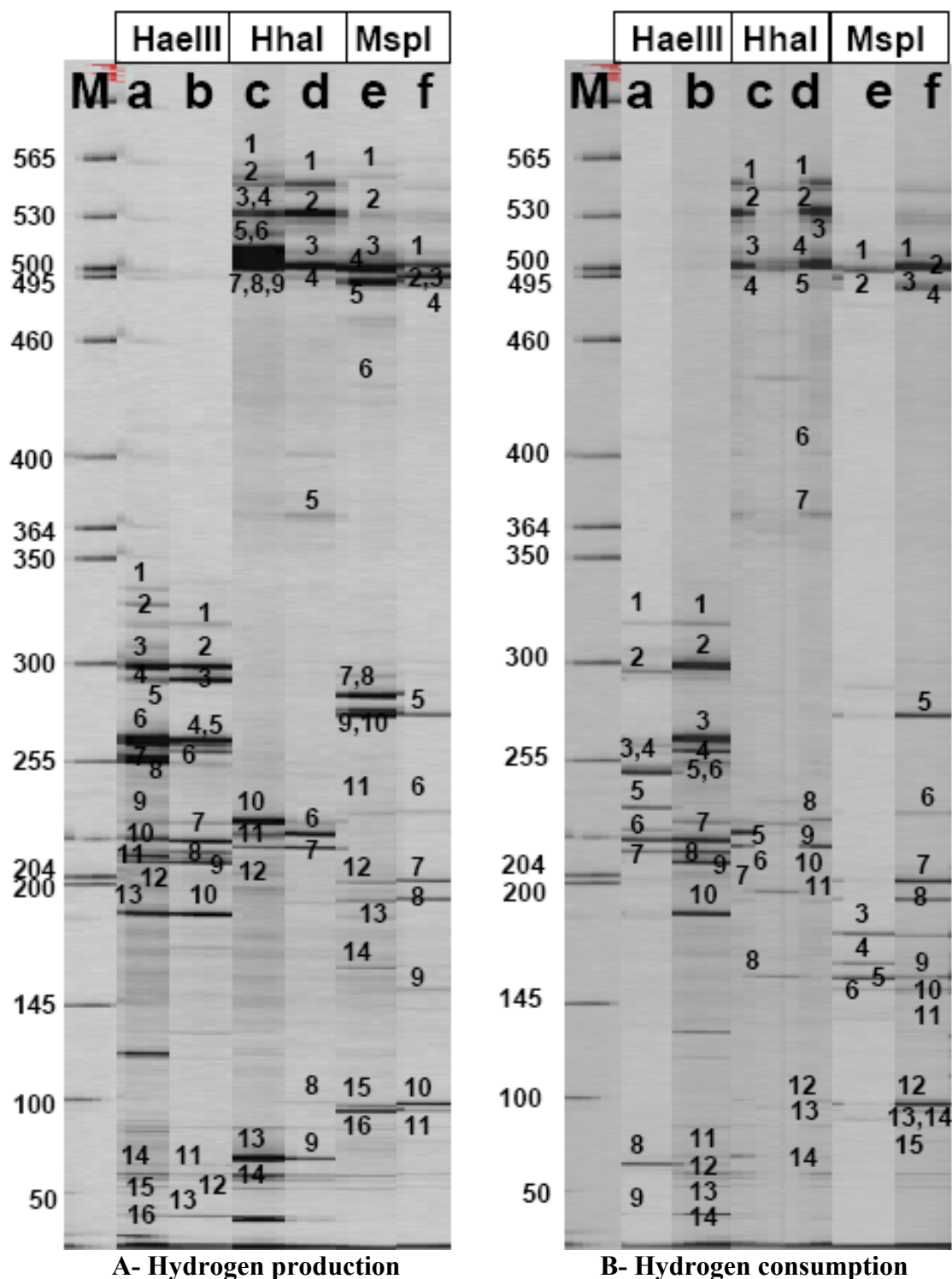


Figure 6.7 Bacterial community profiles as a function of the presence of LA in studies of  $H_2$  production (A) and consumption (B) with initial pH 5.0 as detected by terminal restriction fragment length polymorphism of PCR-amplified 16S rRNA gene. (Lanes a, c, and e = control samples; lanes b, d, and f = LA-treated samples). Numbers on lanes indicate the bands identified.

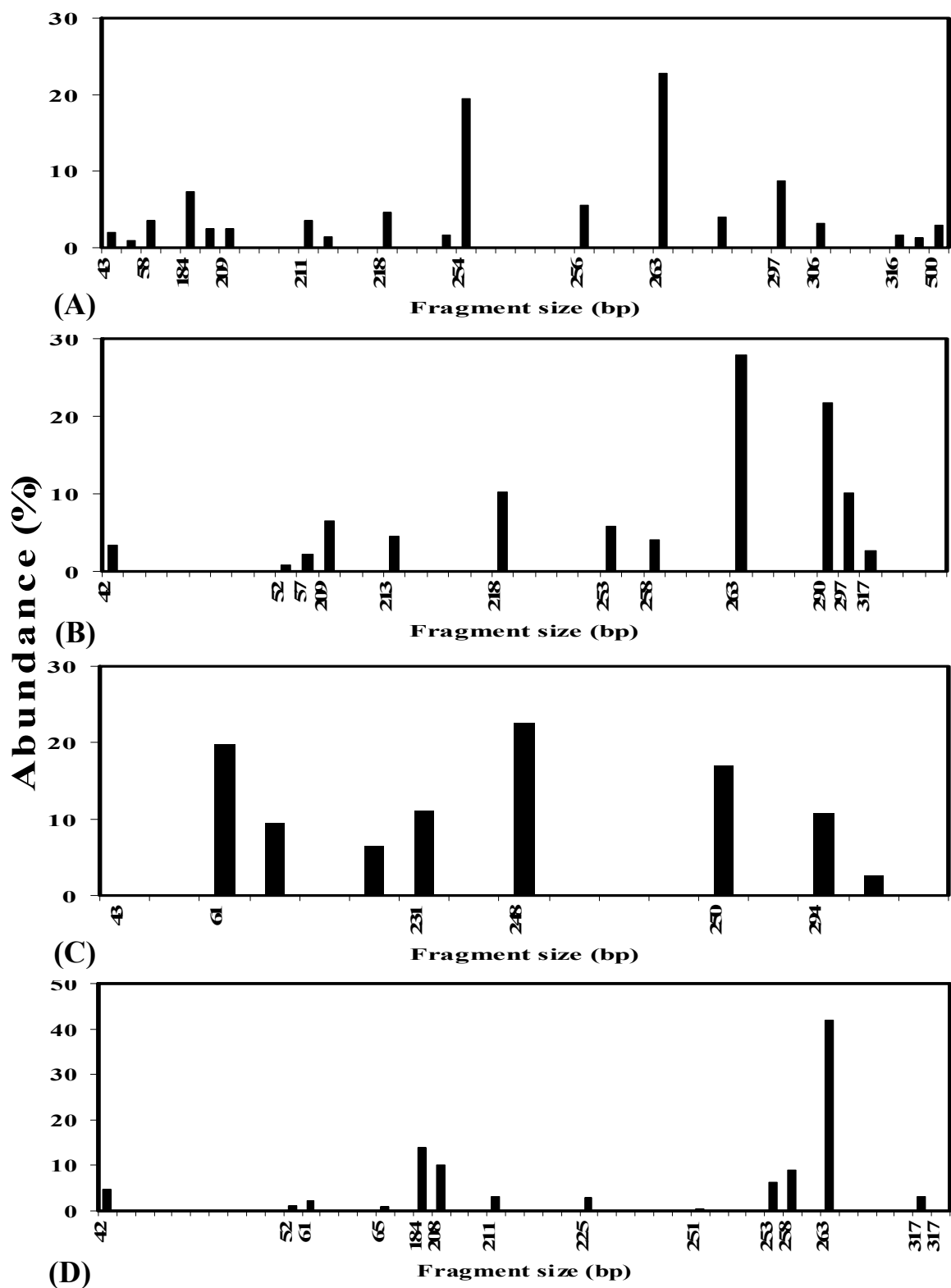


Figure 6.8 Comparison of (A) glucose control, (B) glucose-fed plus LA-treated culture in  $H_2$  production experiment, (C) control (fed only  $H_2$ ) and (D)  $H_2$ -fed plus LA-treated cultures in  $H_2$  consumption experiment based on (bp) of the enzyme *Hae* III.

*Anaeroplasma abactoclasticum* (band 10; 4.63%); *Eubacterium ruminantium* (band 5; 4%); *Rubrobacter xylanophilus* (band 9; 4%); *Clostridium butyricum* (band 3; 3%); and *Geobacter lovely* (band 1; 1.3%).

Experimental (glucose-fed plus LA-treated) samples from the H<sub>2</sub> production experiment (Lane b in Figure 6.7A) were dominated by *Bacteroides*, *Flavobacteria*, *Syntrophus aciditropicum* (band 4; 28%); *Clostridium spiroforme* (band 3; 10%); *Clostridium* sp. and *Geobacillus* (band 7; 10%); *Clostridium polysaccharolyticum* (band 9; 7%); *Clostridium botulinum* (band 6; 6%); *Bacteroides* and *Actinobacillus distasonis* (band 5; 4%); *Citrobacter*, *Cytophaga*, *Enterobacter*, *Erwinia*, *E. coli*, and *Klebsiella* (band 13; 3.4%); *Clostridium thermocellum* (band 1; 2.7%).

The control samples from the H<sub>2</sub> consumption experiment (Hae III: Lane a in Figure 6.7B) were dominated by *Clostridium botulinum* and *Bacillus cereus* (band 5; 23%); *Prochlorococcus marinnis* (band 8; 20%); *Victivallis vadensis* (band 3; 17 %); *Bacillus* sp. (band 5; 11%); *Clostridium* sp. (band 7; 11%); *Rubrobacter xylanophilus* and *Paennibacillus chondroitinus* (band 6; 9%); *Clostridium* sp. (band 4; 7%); and *Clostridium thermocellum* (band 1; 3%).

LA-treated samples from the H<sub>2</sub> consumption experiment (HaeIII: Lane b in Figure 6.7B) were dominated by *Bacteroides* and *Rubrobacter xylanophilus* (band 3; 42%); *E. coli* (band 10; 14%); *Flavobacterium* (band 9; 10%); *Actinobacillus* (band 4; 9%); *Bacillus* sp. (band 5; 6%); *Citrobacter*, *Clostridium thermocellum*, *Enterobacter*, and *Flavobacterium* (band 14; 5%); *Clostridium* sp. (band 1; 3%); *Bacteroides*, *Propionibacterium acnes*, and *Thermoanaerobacter* (band 8; 3%); *Bacteroides*, *Butyrivibrio*, *Clostridium purinolyticum*, *Cytophaga fermentans*, and *Klebsiella* (band 7;

3%); *Bacteroides* and *Lactobacilus* (band 11; 2%); *Parabacillus*, (band 13; 1%); and *Syntrophus aciditrophicum* (band 12; 0.9%).

Analysis of the species in the samples from the H<sub>2</sub> production study (Figures 6.7 and 6.8) reveals three groups of bacteria: 1- Species which were not affected by LA, 2- Species which were eliminated by LA, and 3- Species which grow in LA samples:

1- Species not affected by the presence of LA in the H<sub>2</sub> production samples included *Anaeroplasma abactoclasticum*, *Bacteroides*, *Candidate division*, *Carnobacterium*, *C. botulinum*, *C. butyricum*, *C. spiroforme*, *C. thermocellum*, *Dechlorisoma suilla*, *Eikenella* sp, *Flavobacterium*, *Methyloarcula terricola*, *Parabacteroides johnsonii*, *Thermosiphon africanus*, *Thioalkalivibrio* sp, and *Victivallis vadensis*.

2- Species that were eliminated from LA-treated samples in the H<sub>2</sub> production experiment included *Acidaminococcus* sp, *Acinetobacter calcoaceticus*, *Bacillus cereus*, *Bacillus marismortui*, *C. purinolyticum*, *Comamonas terrigena*, *Dechlorimonas*, *Desulfothiovibrio peptidovorans*, *Fusobacterium nucleatum*, *Heliobacterium modesticaldum*, *Lactococcus lactis*, *Meiothermus ruber*, *Methylophaga buryaticum*, *Moorella thermoacetica*, and *Parabacteroides merdae*.

3- Species that appeared and showed enhanced growth in LA-treated samples from the H<sub>2</sub> production experiment included: *Actinobacillus succinogenes*, *Aranicola proteolyticus*, *Bacteroides*, *Citrobacter*, *Coxiella burnetii*, *Cytophaga marinoflava*, *Enterobacter pyrinus*, *Erwinia amylovora*, *E. coli*, *Geobacillus*, *Methylophaga thalassica*, *Microscilla*, *Propionibacterium acnes*, and *Syntrophus aciditrophicus*.

Analysis of the species in microbial samples from the H<sub>2</sub> consumption study reveals the following groups of bacteria: 1- Species not affected by treatment with LA included



*Bacillus*, *Bacteroides*, *Clostridium*, *Paenibacillus*, *Parabacteroides johnsonii*, *Rubrobacter xylanophilus*, and *Victivallis Vadeness*; 2- Species that disappeared from LA-treated samples in the H<sub>2</sub> consumption experiment included *Bacillus alcalophilus*, *Bacillus benzoovorans*, *Dethiosulfovibrio peptidovorans*, *Fusobacterium gonidiaformans*, and *Prochlorococcus marinus*; and 3- Species that appeared and grew in LA-treated samples in the H<sub>2</sub> consumption study included *Bacteroides*, *Brevibacillus agri*, *Butyrivibrio crosstus*, *Cadidate division*, *Capnocytophaga*, *Citrobacter*, *C. spiroforme*, *Coxiella burnetii*, *Curacaobacter baltica*, *Cytophaga*, *Dechlorisoma suilla*, *Desulfatibacillum alkenivorans*, *Eikenella*, *Entrobacter*, *Erwinia*, *E. coli*, *Flavobacterium*, *Geobacillus*, *Klebsiella*, *Halobacillus litoralis*, *Methylobacillus flagellatum*, *Mycobacterium* sp., *Myxococcus Xanthus*, *Propionibacterium acnes*, *Syntrophus aciditrophicus*, *Thermoanaerobacter*, *Thermosipho africanus*, *Thioalkalivibrio*, and *Victivallis vadensis*.

Some species responded similarly to LA in the H<sub>2</sub> production and consumption studies. *Citrobacter*, *Coxiella burnetii*, *Cytophaga*, *Desulfatibacillum*, *Entrobacter*, *Erwinia*, *E. coli*, *Flavobacterium*, and *Geobacillus* appeared and grew in LA-treated samples in both H<sub>2</sub> production and consumption experiments. Species such as *Bacteroides*, *Clostridium*, *Parabacteroides johsonii*, and *Victivallis vadensis* were not affected by the treatment with LA in both types of experiments. *Fusobacterium* was eliminated from LA-treated samples in both studies.

### **6.3.8 Principal components analysis of the microbiological data**

Principal components analysis (PCA) on T-RFLP profiles with *Hae* III, *Hha* I, and *Msp* I enzymes calculated scores for the data sets of the three enzymes (Figure 6.9). Two

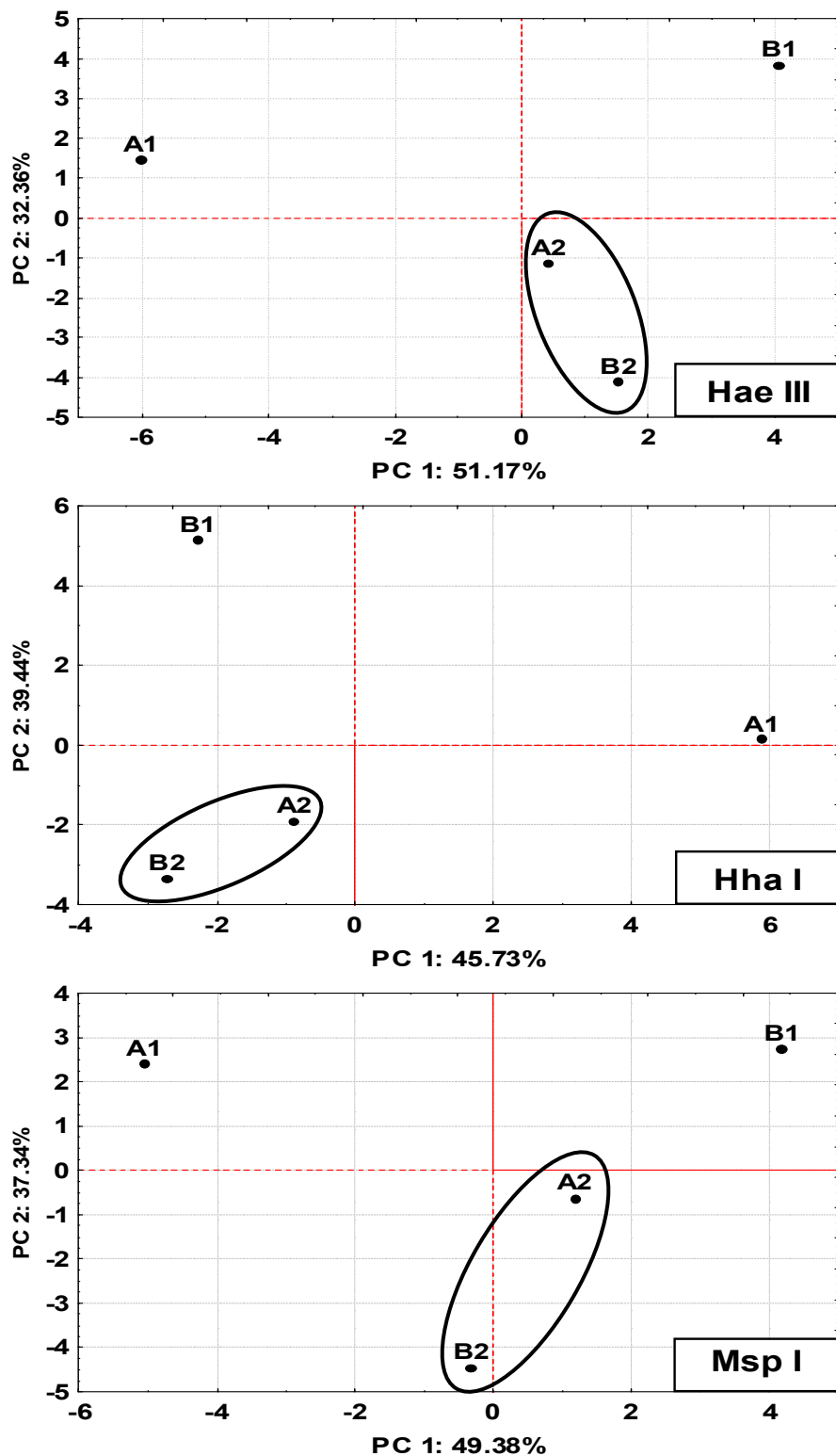


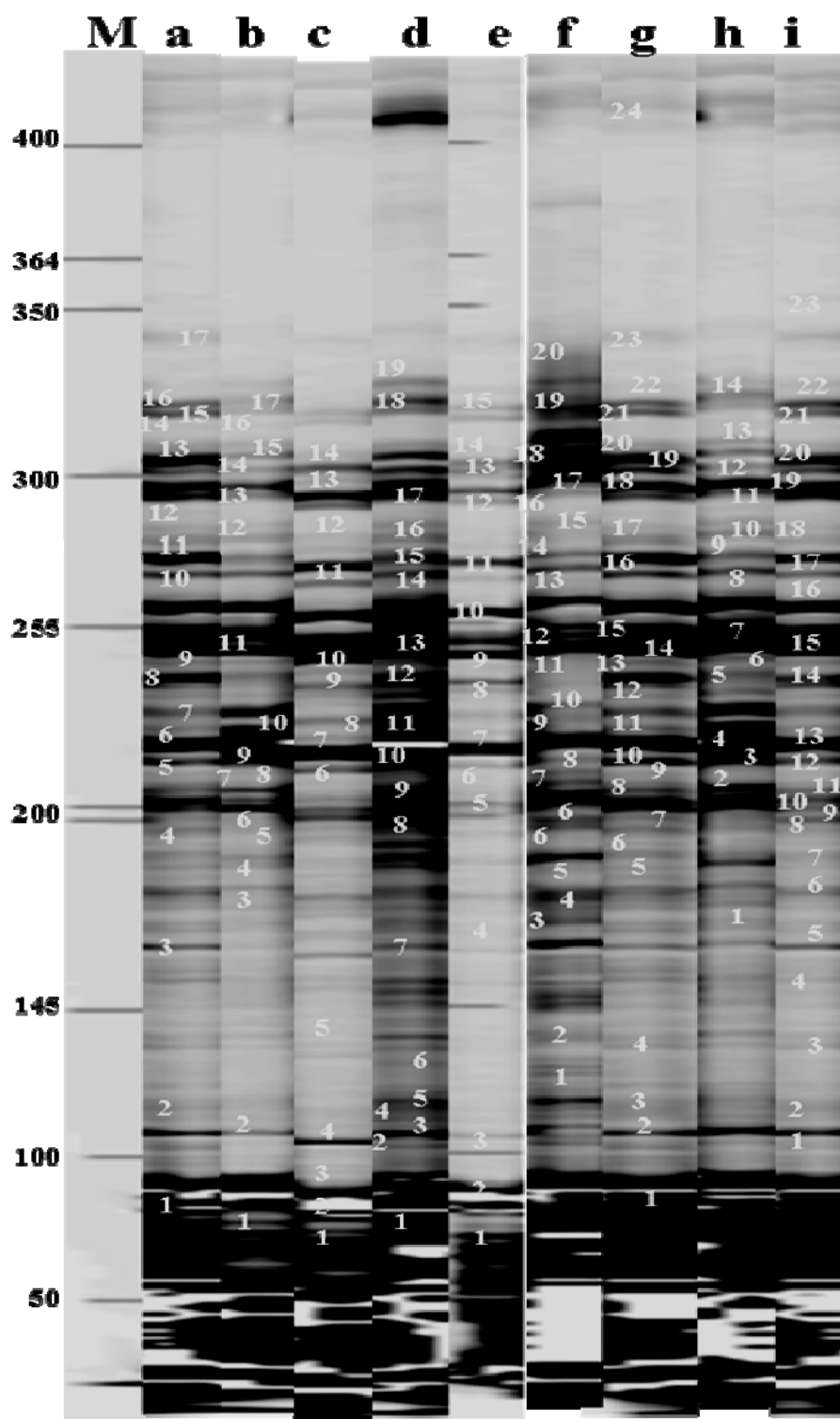
Figure 6.9 PCA analysis of molecular weight data set for bacteria bands from T-RFLP gel image.(A1 and A2 = glucose control and glucose-fed plus LA-treated cultures from the H<sub>2</sub> production experiment. B1 and B2 = H<sub>2</sub> control (fed only H<sub>2</sub>) and H<sub>2</sub>-fed plus LA-treated cultures from the H<sub>2</sub> consumption experiment).

principal components (PCs) explained 83 % of the total variability in the *Hae* III data set, 2 PCs explained 85 % of the total variability in the *Hha* I data set, and 2 PCs explained 86.72% of the total variability in the *Msp* I data set. PCA score maps showed that for each of the enzymes there were distinct differences between the samples from cultures with and without LA under the conditions examined.

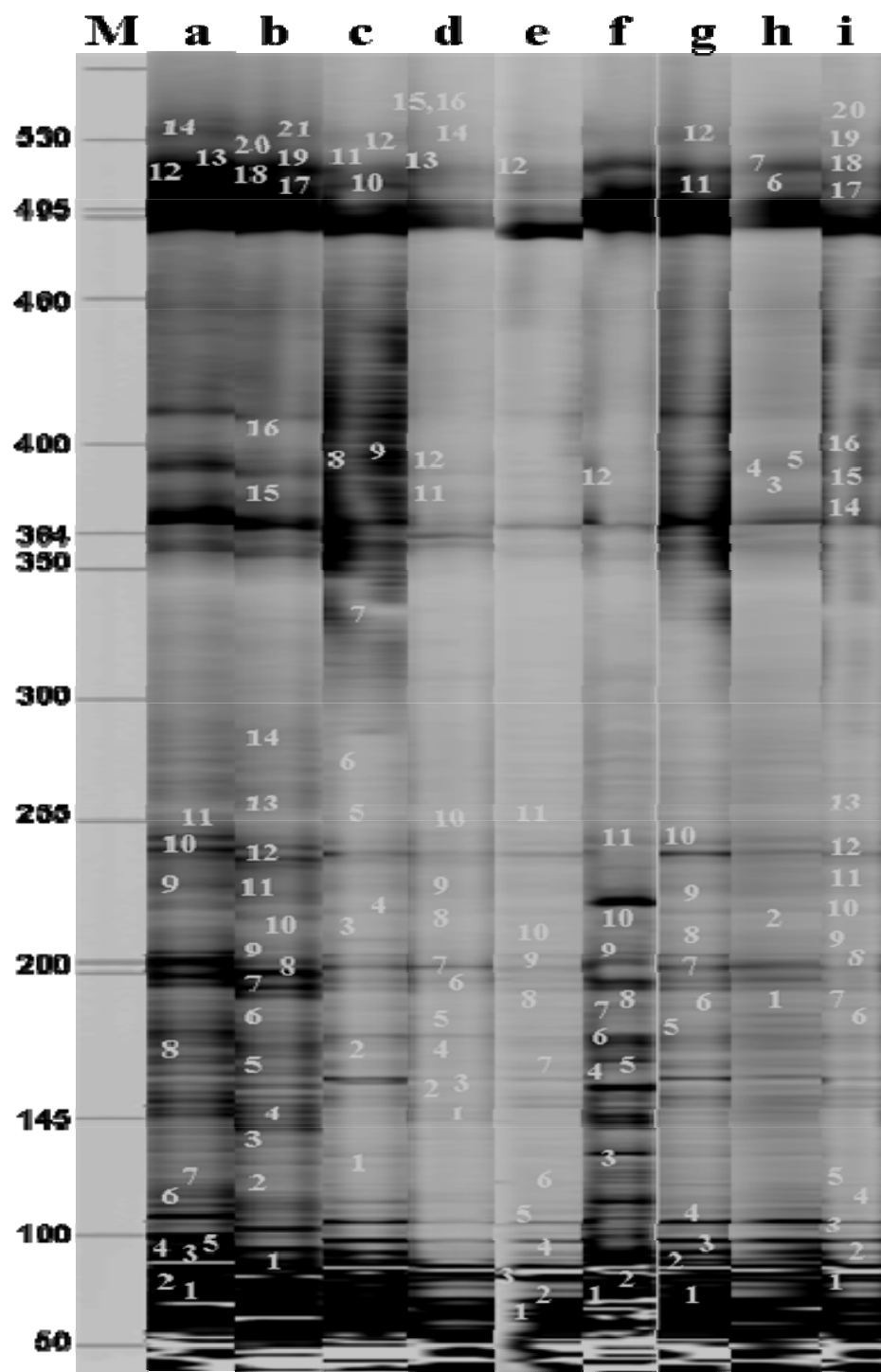
LA-treated samples from experiments of H<sub>2</sub> production (A2) and H<sub>2</sub> consumption (B2) behaved similarly and clustered together on the score map of PC1 and PC2 for all the enzymes used in T-RFLP (Figure 6.9). Notice the small differences in the scores of A2 and B2, especially on PC1, for all of the three enzymes. At the same time, control samples from the two experiments (A1 and B1) were different from each other; in particular, there were large differences between the scores for A1 and B1, especially on PC1 for all of the three enzymes. Moreover, the control samples (A1 and B1) differed from their corresponding LA-treated samples (A2 and B2). Notice that the control samples (A1 and B1) are located in the upper half (the positive side of PC1) on the score maps for all three enzymes, whereas LA-treated samples (A2 and B2) are located in the lower half (the negative side of PC1) of the score maps (Figure 6.9).

### **6.3.9 Microbiological characterization of VFAs degradation in the presence of LA**

The composition of bacterial communities in cultures incubated with lactate, acetate, propionate, or butyrate, with and without LA, was analyzed using the T-RFLP method. The bands detected are shown in the gel images (Figure 6.10), and the relative abundance is shown in Figure 6.11.

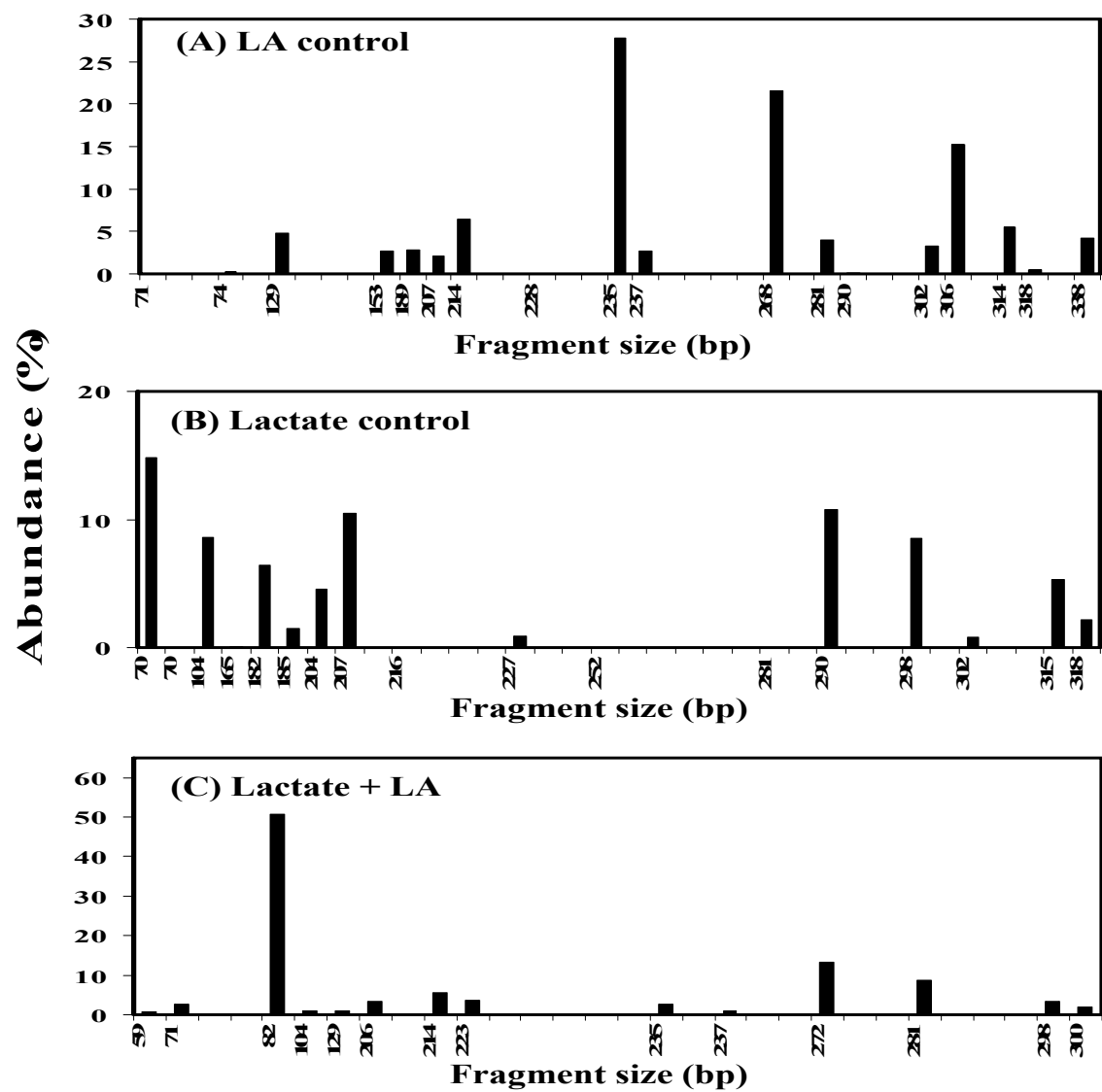


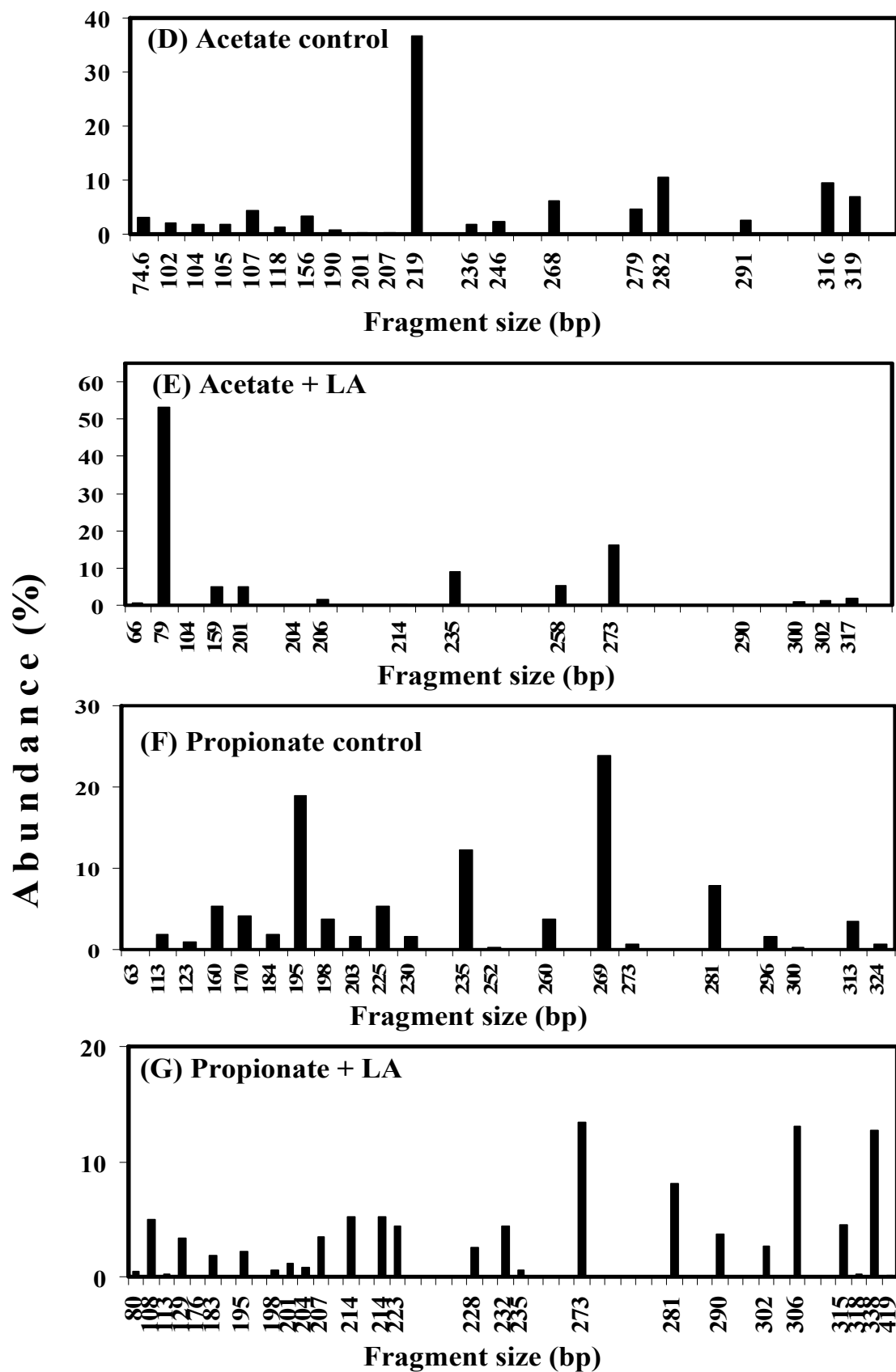
A- Enzyme *Hae* III



### B- Enzyme *Hha* I

Figure 6.10 Bacterial community profiles as a function of exposure to LA in studies of VFAs degradation studies. Lane a = control culture. Lanes b, d, f, and h = controls (without LA) of lactate, acetate, propionate, and butyrate, respectively. Lanes c, e, g, and i = LA-treated cultures of lactate, acetate, propionate, and butyrate, respectively. Numbers on lanes indicate the bands identified.





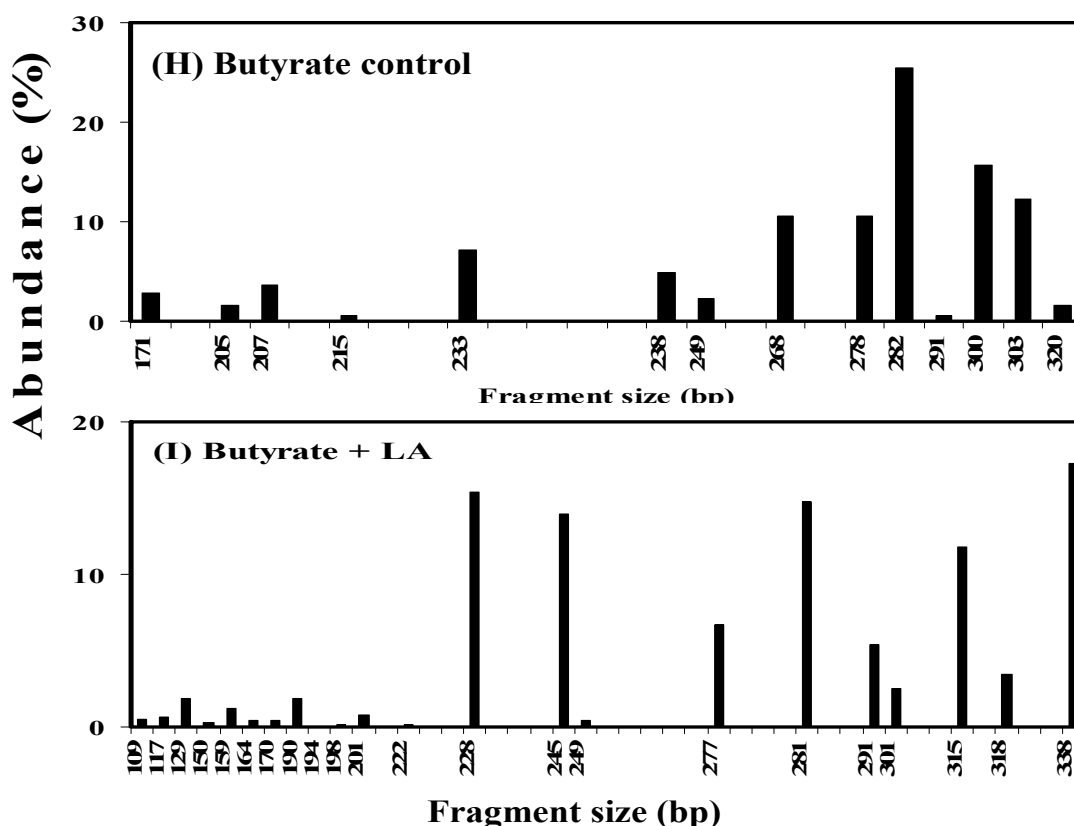


Figure 6.11 Comparison of (A) LA control, (B) lactate control, (C) lactate plus LA, (D) acetate control, (E) acetate plus LA, (F) propionate control, (G) propionate plus LA, (H) butyrate control, and (I) butyrate plus LA incubated cultures in studies of VFAs degradation; based on fragment size (bp) of the enzyme *Hae* III.

The characterization of different microbial communities demonstrated the presence of distinctive bands. The LA-control culture samples (Lane a in Figure 6.10 and Figure 6.11A) were dominated by *Bacillus* sp. (bands 8, 9, 14, 15 and 16; 50%); *Bacteroides* sp. (band 10; 21%); *Morella thermoacetica* (band 14; 15%); *Clostridium* sp. (band 6; 6 %); and *Geobacter* (band 17; 4%).

In the control and LA-treated samples from the lactate degradation experiment (Lanes b and c in Figure 6.10 and Figure 6.11B and C), the lactate control samples were dominated by *Thermanaerovibrio acidaminovorans* (band 2; 9%); *Eubacterium* sp. (band 8; 14%); and *Clostridium* sp. (bands 8, 9, 10, 14, and 15; 28%). The LA-treated samples



were dominated by *Azoarcus* sp. (band 3; 49%); *Clostridium* sp. (band 11; 12.89%); *Fusobacterium* sp., *Lactobacillus* sp., and *Cetobacterium* sp. (band 12; 8%); *Eubacterium* sp. (band 6 and 8; 7%); *Acidimicrobium* sp. (band 7; 5%); and *Acetobacter* sp. (band 13; 3%).

In control and LA-treated cultures from the acetate degradation experiment (Lanes d and e in Figure 6.10 and Figure 6.11D and E), the acetate control samples were dominated by *Geobacter* sp. (bands 10; 37%); *Cetobacterium* sp. (band 16; 10.49%); *Clostridium* sp. (band 7 and 13; 10%); *Butyrivibrio* sp. (band 13; 7%); and *Thermanaerovibrio* sp. (band 19; 4%). LA-treated samples from the acetate degradation experiments were dominated by *Syntrophobacter* sp. (band 2; 53%); *Butyrivibrio* sp. and *Clostridium* sp. (band 11; 16%); *Bacillus* sp. (bands 1, 9, and 15; 11%); and *Bifidobacterium* sp. (band 10; 5%).

In control and LA-treated cultures from the propionate degradation experiment (Lanes f and g in Figure 6.10 and Figure 6.11F and G), the propionate control samples were dominated by *Fusobacterium* sp. (band 14; 24%); *Lactobacillus* sp. (band 11; 12%); *Cetobacterium* sp. (band 16; 8%); *Flavobacterium* sp. and *Bacteroides* sp. (band 13; 4%); and *Clostridium* sp. (band 18; 0.3%). LA-treated samples from the propionate degradation experiments were dominated by *Bacillus* sp. (bands 5, 12, 15, 19, 20 and 22; 24%); *Clostridium* sp. (band 10, 11, 12, 16, 18, and 19; 25%); and *Geobacter* sp. (bands 11, 12, 15, 16, and 23; 13 %); and *Thermanaerovibrio* sp. (band 2; 5%).

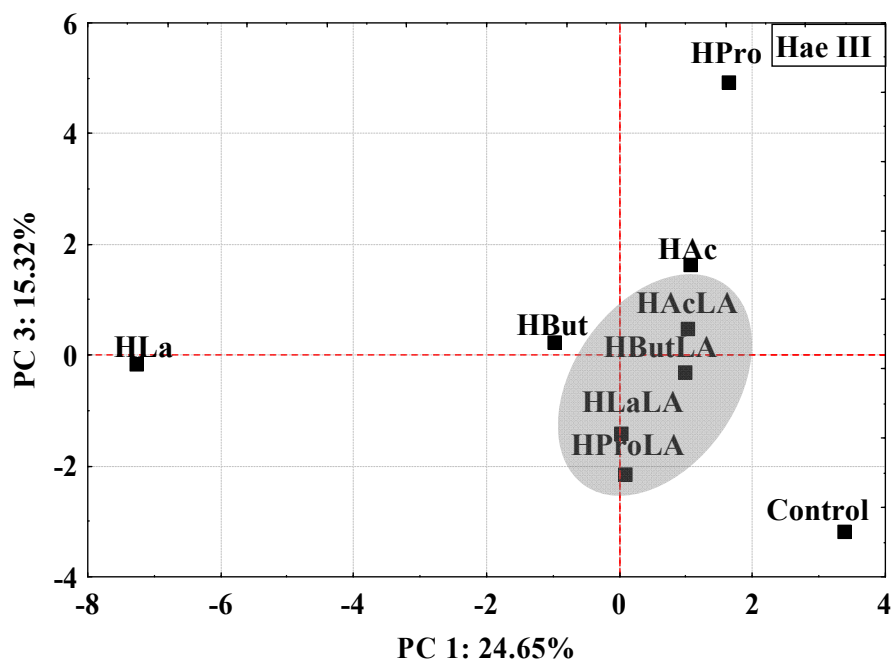
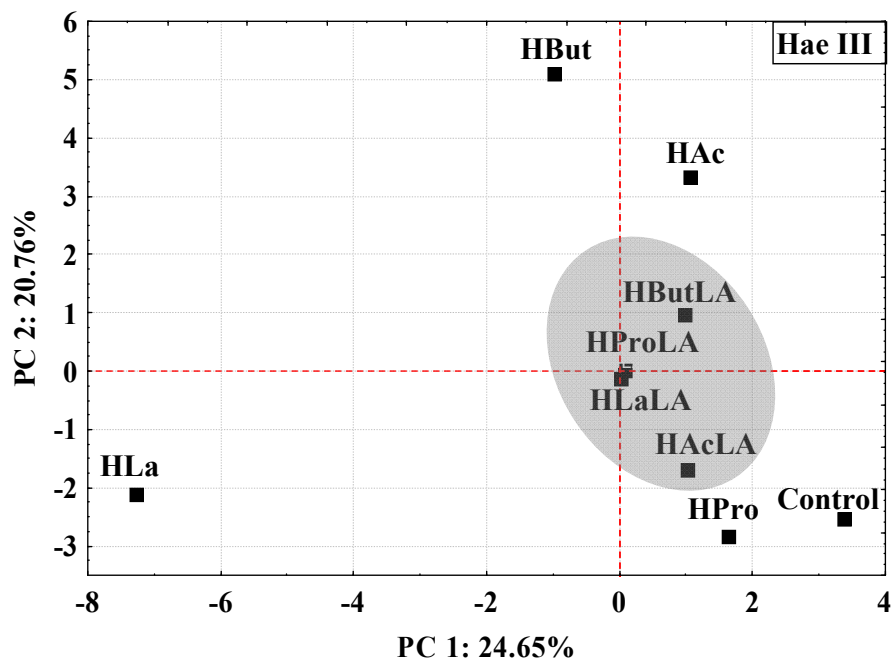
In control and LA-treated samples from the butyrate degradation experiment (Lanes h and i in Figure 6.10 and Figure 6.11H and I), the butyrate control samples were dominated by *Eubacterium* sp. (band 10; 25%); *Enterococcus* sp. (band 12; 15%);

*Clostridium* sp. (band 9 and 13; 22%); *Lactobacillus* sp., (band 7; 10%); *Acetobacter* sp. and *Geobacter* sp. (band 5; 7%). LA-treated samples from the butyrate degradation experiments were dominated by *Clostridium* sp. (bands 11, 12, 13, 15, 17, and 20; 31%); *Acetobacter* sp. (band 13; 19%); *Cetobacterium* sp. (band 18; 18%); *Eubacterium* sp. (band 14; 17%); *Bacillus* sp. (band 21; 14%); and *Butyrivibrio* sp. (band 17; 8%).

#### **6.3.10 Principal components analysis of the microbiological data**

Principal components analysis (PCA) on T-RFLP profiles calculated scores for the *Hae* III enzyme data set (Figure 6.12). Three principal components (PCs) explained 61% of the total variability in the *Hae* III data set. Each PC accounted for a different proportion of the variability in this data set: PC1 (24.65%), PC2 (21%), and PC3 (15%). The larger number of PCs obtained in the analysis indicates that there were no strong correlations among the data sets of the individual LCFAs. PCA score maps showed distinct differences, however, between the samples of cultures treated with LA those that were not exposed to LA under the different conditions examined.

LA-treated samples from studies of lactate, acetate, propionate, and butyrate degradation behaved similarly and clustered together on the score maps of PC1, PC2, and PC3 (Figure 6.12; shaded areas). The positions for the control samples were more widely scattered. Notice the differences in the position of the control and LA-treated samples on the score maps in Figure 6.12.



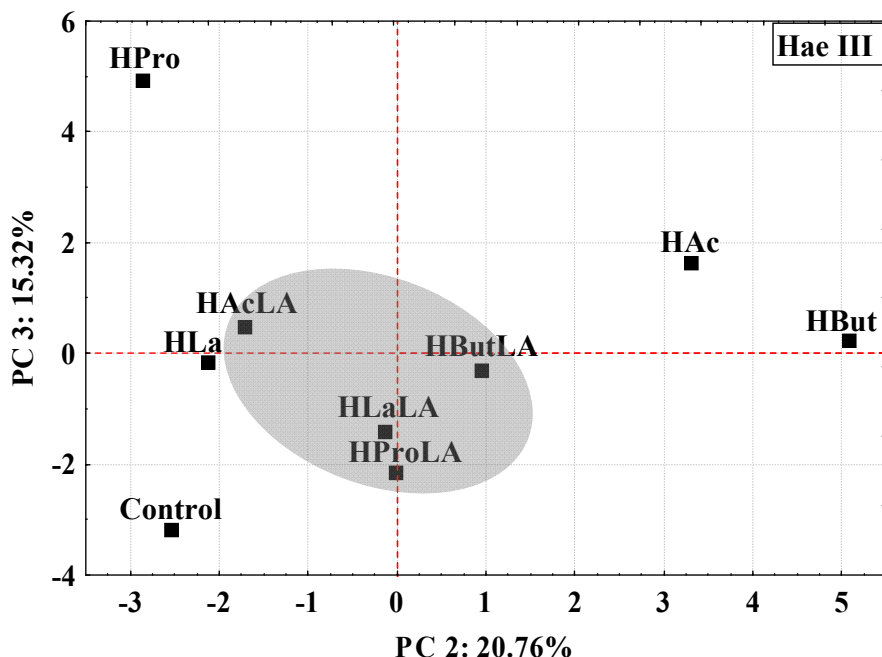


Figure 6.12 PCA of molecular weight data sets for bacteria bands from T-RFLP gel images for lactate, acetate, propionate, and butyrate degradation studies in the presence or absence of LA. (HLa, HAc, HPro, and HBut = control samples of lactate, acetate, propionate, and butyrate-fed culture without LA. HLaLA, HAcLA, HProLA, and HButLA = LA-treated samples of lactate, acetate, propionate, and butyrate-fed culture).

## 6.4 DISCUSSION

The populations and activities of the various trophic groups within the anaerobic consortia is a function of the environmental and stress conditions. The microbial community composition of active acidogens involved in the carbon flow during  $H_2$  fermentation using heterogeneous mixed culture is still largely unresolved. To date no published study has quantified the changes in the proportions and activities of acidogens, acetogens and methanogens in  $H_2$ -producing mixed culture under the stresses of chemical inhibitors or changing environmental conditions. The presence of LCFAs and low pH have been shown to affect the flow of electron equivalents and re-direct a larger proportion towards  $H_2$ , yet their effects on the different trophic groups in  $H_2$ -producing

mixed culture have not been evaluated. This research quantified the effects of LA (2,000 mg L<sup>-1</sup>) and low pH (5.0) on the kinetics and microbiology of mesophilic H<sub>2</sub>-producing mixed anaerobic culture in dark fermentation.

The results demonstrate that the effects of the stress imposed by low pH on the activity and abundance of the different trophic groups were variable within the culture community. Notice low pH alone was insufficient to suppress H<sub>2</sub> consumers or to increase the yield of H<sub>2</sub>. However, adding 2,000 mg L<sup>-1</sup> LA significantly decreased the activity and abundance of active H<sub>2</sub>-consuming microorganisms which, in turn, decreased H<sub>2</sub> consumption and increased H<sub>2</sub> yield. The results are consistent with previously reported findings that pH affects microbial populations and their metabolic activities (Wang *et al.*, 2007); particularly, the dynamic state of H<sub>2</sub>-producing and -consuming microbial populations (Goodwin *et al.*, 1988). These researchers concluded that the pH-dependent accumulation of organic fatty acids and alcohols affects the dynamics of H<sub>2</sub> metabolism.

Monitoring the microbial community composition during H<sub>2</sub> consumption experiments elucidated H<sub>2</sub> consumption by non-methanogenic microorganisms. *Clostridia* were detected in all control and LA-treated cultures from the H<sub>2</sub> production and consumption experiments as well as the lactate, acetate, propionate, and butyrate degradation experiments. Noticed the abundance of *Clostridium* sp. (Table 6.6) was variable with the type of substrate. It is evident that LA increased the percentage of biomass comprised of *Clostridium* sp. in glucose-, acetate-, propionate-, and butyrate-fed cultures whereas it decreased this percentage in H<sub>2</sub>- and lactate-fed cultures. The kinetic assay indicated that the percentage of biomass concentration made up of active H<sub>2</sub> degraders increased after

the addition of LA while the T-RFLP profiles of LA-treated culture in the H<sub>2</sub> consumption experiment indicated that the percentage of *Clostridium* sp. decreased. This is consistent with the findings of Wiegel *et al.* (2006) that only several *Clostridium* species such as *C. acidisoli* and *C. pasteurianum* are acid-tolerant and able to grow at low pH (~3.6).

Table 6.6 Effects of LA on the percent *Clostridium* sp. observed in T-RFLP profiles in various substrate degradation experiments.

<u>Substrate</u>	<u>Percentage of the <i>Clostridium</i> sp. biomass concentration of specific substrate degraders</u>	
	<u>Control</u>	<u>LA-treated</u>
Glucose	11.76	35.37
H <sub>2</sub>	42.55	<b>11.00</b>
Lactate	27.85	<b>12.89</b>
Acetate	9.51	16.15
Propionate	0.31	23.53
Butyrate	22.22	31.44

Note: Bold font numbers indicate a decrease in biomass concentration compared to control.

The results suggest that species other than *Clostridia* play a significant role in H<sub>2</sub> consumption as well. For example, some of the identified species, such as *Moorella thermoacetica* (Drake and Daniel, 2004; Goßner *et al.*, 2008), *Eubacterium*, *Acetobacterium*, and *Syntrophobacter* (Drake, 1994), are known homoacetogens (see sections 2.2.3, 2.2.4 and Table 2.3). *Acetobacterium* sp. are Gram-positive non-spore-forming homoacetogenic bacteria (Schink and Bomar, 1991). These microorganisms derive more thermodynamic metabolic efficiency by consuming H<sub>2</sub> than producing it when they grow on multiple carbon compounds (Marchaim, 1992). (Notice the difference in  $\Delta G$  value in Eq. 14 compared to Eq. 10, 11, 12, and 13 in Table 2.1). Moreover, sulfate-reducing bacteria such as *Desulfotomaculum* genus can grow autotrophically on H<sub>2</sub>/CO<sub>2</sub> in the absence of sulfate (Goorissen *et al.* 2004; Min and Zinder 1990). Although

*Thermoanaerobacterium* sp. are H<sub>2</sub> producing bacteria (Ueno *et al.*, 2001) they formed approximately 3% of the LA-treated culture in H<sub>2</sub> consumption experiment; however, no homoacetogenic activity has been reported to date.

The flux balance analysis (FBA) did not predict homoacetogenic H<sub>2</sub> consumption in LA-treated culture from the H<sub>2</sub>-production experiment after both the first and second glucose injections. Similarly, the FBA was unable to predict homoacetogenic H<sub>2</sub> consumption in the control culture after the first glucose injection; however, it did predict increasing homoacetogenic activity after the second glucose injection. On Days 7 and 8, homoacetogens were able to consume 0.14 and 0.42 mol H<sub>2</sub> per mol of glucose injected. The FBA revealed that the solventogenic reaction consumed almost the same quantity of H<sub>2</sub> in the controls and the LA-treated cultures.

The greater diversity observed for bacteria in the LA-treated cultures from the H<sub>2</sub> consumption assay in comparison to the diversity in the control samples (received H<sub>2</sub> without LA) is likely due to methanogenic inhibition in the LA-treated cultures. Upon such inhibition, H<sub>2</sub> was available as an excellent electron donor for other microorganisms such as homoacetogens, sulfate and iron reducers, denitrifiers, and aromatic compound oxidizers (Lee *et al.*, 2009). Under normal conditions, these microorganisms are less competitive than methanogens; however, they were dominant and the percent biomass concentration increased after adding LA as demonstrated by the results of the activity assay (Table 6.1) and T-RFLP profile from the H<sub>2</sub>-consumption experiment (Figure 6.7B and Figure 6.8).

Upon decreasing the pH from 7.6 to 5.0 and then adding LA, the percentage of acidogens and acetogens (relative to the total active biomass) increased from 59% (pH

7.6) to 63% (pH 5.0) and then to 70% (LA), respectively. Concurrently, the percentages of acetate- and H<sub>2</sub>- consumers decreased from 41% (pH 7.6) to 37% (pH 5.0) and then to 30% (LA), respectively. The results of the acetate- and H<sub>2</sub>-consuming activity at pH 7.6 are in agreement with previously reported data. Dolfig and Bloemen (1985) reported that the specific hydrogenotrophic activity is higher than the specific activity of acetate consumers by a factor of 10. In comparison, in this study, the factor was 12 at pH 7.6 and 22 at pH 5.0.

The change in culture composition after lowering the pH and adding LA is believed to be due to increased differential inhibition imposed on the various trophic groups, change in substrate composition, bacterial decay rates, and maintenance energy requirements (Criddle, 1993; Strydom *et al.*, 2001). LA caused 75 % of the 99% reduction in the H<sub>2</sub> specific consumption rate while pH resulted in only a 24% reduction. Post hoc analyses (Tukey's multiple comparison test at 95% confidence interval; Box *et al.*, 1978) were used to compare maximum specific H<sub>2</sub> utilization rates under the different conditions of increasing stress due to lowering the pH and the addition of LA. The maximum specific H<sub>2</sub> utilization rates of the cultures at pH 7.6 and 5.0 were significantly different from each other, and they were significantly different from the maximum H<sub>2</sub> specific utilization rate of the LA-treated culture. Changes due to lowering the pH are consistent with the findings of Goodwin and Zeikus (1987). They reported that the optimum pH for H<sub>2</sub>-consuming microorganisms is 5.6 and that decreasing the pH to 4.8 and 3.5 reduced the H<sub>2</sub> consumption rate by only 13% and 28%, respectively (Table 6.7). In fact, decreasing the pH from 5.6 (optimum for H<sub>2</sub>-consumers) to 4.8 (very close to 5.0, the optimum for



H<sub>2</sub> producers) decreased the H<sub>2</sub> consumption rate by only 13%; therefore, reducing the pH from 7.6 to 5.0 in this study did not present a strong inhibitory stress to H<sub>2</sub> consumers.

Table 6.7 Influence of pH on H<sub>2</sub> consumption rate in anaerobic mixed culture.

pH	3.5	4.2	4.8	5.6	6.2	7.0
H <sub>2</sub> consumption rate ( $\mu\text{mol L}^{-1} \text{h}^{-1}$ )	5.0	5.3	6.0	6.9	4.4	4.2

Note: adapted from Goodwin and Zeikus (1987).

Another example of the impact of LA was on propionate-producing bacteria such as *Clostridium*, *Bacteriodes* and *Fusobacterium* (Border *et al.*, 1987). Although these species were detected in the LA-treated culture and their concentrations were higher than in the control culture, no propionate was detected in LA-treated samples from the H<sub>2</sub> production experiment. These results suggest that LA influenced the metabolic pathways of these microorganisms. All of these changes indicate that the influence of LA on the microbial population, their biological activities, and their metabolic pathways was notable.

The FBA supports the findings of the kinetic study. The FBA model showed no homoacetogenic H<sub>2</sub> consumption in LA-treated culture whereas 0.42 mol H<sub>2</sub>·mol<sup>-1</sup> glucose (18% of the maximum yield) was consumed in the control culture. Therefore, homoacetogenesis was responsible for approximately 60% of the H<sub>2</sub> consumed in the control cultures. The other H<sub>2</sub> sink predicted by the FBA was solventogenesis (R21). Production of isopropanol and propanol is expected because of the dominance of *Clostridium* sp. which can convert acetate, butyrate and H<sub>2</sub> to propanol and butanol when the  $P_{H_2}$  is high (Jones and Woods, 1986). Increasing concentrations of VFAs production

is known to trigger solventogenesis. Many acidogens such as *C. acetobutylicum* (Bahl *et al.*, 1982), *C. butyricum* (Andel *et al.*, 1985), *Clostridium cellobioparum* (Chung, 1976), *C. fallax* (Ueki *et al.*, 1991), and *C. pasteurianum* (Dabrock *et al.*, 1992) can produce H<sub>2</sub>, acetate, and butyrate and shift the metabolic pathways to produce propanol, butanol, and even acetone under increased VFAs concentration and low pH. For example, Gottschalk and Morris (1981) reported that adding 600 mg L<sup>-1</sup> of acetate or butyrate to batch cultures of *Clostridium acetobutylicum* induced a rapid production of alcohols. The concentrations of acetate in the control and LA-treated cultures after the first glucose injection were respectively 706±50 (Day 3) and 936±30 (Day 2) mg L<sup>-1</sup>. On Day 5 (one day after the second glucose injection), acetate concentrations were 1650±40 and 1950±130 mg L<sup>-1</sup> in the control and LA-treated cultures, respectively. Hence, *Clostridium* sp. species could shift their metabolic pathways towards alcohols production.

Other species that were present in high abundance in LA-treated cultures included *Eubacterim*, *Bacillus*, *Geobacter*, and *Butyrivirio*. The heterogeneous spectrum and abundance of the dominant microorganisms observed in LA-treated samples distinguish it from other pretreatments, which produce homogenous cultures with fewer dominating bands.

VFAs degradation depends on syntrophic H<sub>2</sub> transfer where the activity of one species depends on the activity of another species (McInerney and Beaty, 1988). The syntrophic association between acetogens and methanogens depends on the activity of acetate and H<sub>2</sub>-consuming microorganisms and according to Voolapalli and Stuckey (1999), adding acetate consumers improved the degradation of VFAs more than H<sub>2</sub> consumers). LCFAs

are inhibitory to acetoclastic methanogens and their effects on hydrogenotrophic methanogens are notably less (Templer *et al.*, 2006; Palatsi *et al.*, 2010).

The decrease in the rates of VFAs degradation in the LA-treated cultures is, therefore, due to the inhibition of acetoclastic methanogens, the reduced activity of hydrogenotrophic methanogens and H<sub>2</sub>-producing acetogens. Some *Butyrivibrio* sp. have been reported to hydrogenate LA to trans-octadec-II-enoic acid and some species of Gram-negative *Bacillus* can hydrogenate LA to stearic acid (Kemp *et al.*, 1975). The very small degradation rates of acetate, propionate, and butyrate could still support the growth of some syntrophs, particularly LCFAs oxidizers, because they can generate energy by exchanging flux of materials with the environment (McInerney and Beaty, 1988).

Some work has provided evidence that LCFAs inhibit Gram-positive, but not Gram-negative bacteria. The evidence from this work and other studies suggest that some Gram-positive bacteria can survive inhibition by LCFAs. The dominance of *Clostridium* sp. and other Gram-positive species contradicts data from earlier studies. For example, *Butyrivibrio fibrisolvens* was detected in the LA-treated cultures incubated with acetate and butyrate, and in the controls for acetate and propionate. Although *B. fibrisolvens* is classified as gram-negative, Cheng and Costerton (1977) observed under electron microscopy examination that it has a gram-positive-type cell wall.

The kinetic method used to estimate the percentages of the various trophic groups present in the mixed culture was effective as long as appreciable degradation occurred, and successive initial segments of linear degradation curves could be established. The latter requirement was not possible in the case of acetate, propionate, and butyrate degradation in the presence of LA. Therefore, it was not possible to estimate the

percentage of biomass concentration when only approximate specific activity could be determined for the active degraders (obligatory H<sub>2</sub>-producing acetogenic bacteria) of these substrates because of the strong inhibition imposed by the relatively high concentration of LA (2,000 mg L<sup>-1</sup>). Note that the rate of VFAs degradation is proportional to the H<sub>2</sub>-consuming activity (Voolapalli and Stuckey, 1999), which was also inhibited by LA. Generally, the approximate degradation rate of butyrate in LA-treated culture was 45% of the acetate and 90% of the propionate degradation rates. It is apparent that LA alters the general trend that degradation of butyrate is faster than acetate and propionate (Andrews and Pearson, 1965; McCarty, 1963). Another difficulty in monitoring acetate degradation in bottles containing culture samples was the inability to differentiate between injected acetate and acetate that was a by-product of LA degradation. Assays incorporating labeled carbon acetate may be helpful in resolving this issue.

LA decreased the glucose initial degradation rate in the H<sub>2</sub> production experiment by 88% whereas in the kinetic experiment LA reduced the activity of the active glucose degraders by 26%. Such a differential effect might be due to the synergistic effect of the greater concentrations of VFAs produced during the H<sub>2</sub> production experiment compared to the kinetic study. The concentration of glucose (5,000 mg L<sup>-1</sup>) in H<sub>2</sub> production experiments is 5 times its concentration in the kinetic experiment.

LA inhibited the acetate-, propionate-, and butyrate-degrading microorganisms by 99.9% (Table 6.8). The concentration of LA in the current work is 10 times the 50% inhibitory concentration (IC<sub>50</sub>) reported by Kim *et al.* (2004) for acetate and 6 times that reported for propionate degradation (Table 6.8). Similarly, the concentration of LA used

Table 6.8 LA concentrations and degree of inhibition on VFAs degradation in mixed cultures.

Culture	Substrate	Temp (°C)	LA (mg L <sup>-1</sup> )	Degree of inhibition (%)	Reference
Mixed culture	Acetate	37	2,000	99.8	This study
	Propionate	37	2,000	99.9	This study
	Butyrate	37	2,000	99.9	This study
Mixed culture	Acetate	NR	202	50	Kim <i>et al.</i> , 2004
	Propionate	NR	328	50	
Glucose adapted mixed culture	Butyrate	21	100	92	Lalman and Bagley, 2002
Butyrate adapted mixed culture	Butyrate	21	1,000	93.7	Mykhaylovin <i>et al.</i> , 2005
	Butyrate		45	50	
Acclimated granular sludge	Acetate	35	185-208	50	Shin <i>et al.</i> , 2003
	Propionate		255-353	50	
Mixed culture	Glucose	37	350	50	Lalman and Komjarova, 2004

Note: NR = not reported

in this research is 20 times the concentration of LA used with glucose- and 2 times the concentration of LA used with butyrate-adapted mixed cultures to inhibit butyrate degradation (Lalman and Bagley, 2002; Mykhaylovin *et al.*, 2005). Inhibition of acetoclastic methanogens is correlated with increased LCFAs concentration (Shin *et al.*, 2003).

Furthermore, the pH (5.0) used in this study is lower than that in the studies cited, and there are synergistic effects with other VFAs produced from glucose in this study. The accumulation of acetate in the H<sub>2</sub> production experiments and its low degradation rate (0.002±0.006 mg COD L<sup>-1</sup>·mg·VSS<sup>-1</sup>·d<sup>-1</sup> measured in the kinetic assay) indicate that LA inhibited the acetoclastic methanogens. LA is reportedly inhibitory to butyrate- and propionate-degrading acetogenic bacteria (Lalman and Bagley, 2002; Kim *et al.*, 2004; Mykhaylovin *et al.*, 2005). Butyrate and propionate also accumulate because their degradation to acetate is thermodynamically impossible under the high hydrogen partial

pressure ( $P_{H_2}$ ) and LA-inhibited methanogenesis ( $\Delta G^{\circ} = + 48.30$  and  $+ 71.67$  kJ/mol, respectively) (McCarty and Smith, 1986).

The principal components analysis (PCA) of the T-RFLP profile data sets from the  $H_2$ -production and –consumption experiments and the VFAs degradation studies showed the same trend. In these studies, the LA-treated cultures clustered together indicating their similarity. Conversely, the control cultures were separated from each other and from the LA-treated cultures. The scattered placements indicate differences amongst the control cultures and also differences between controls and the LA-treated cultures. The PCA findings from the T-RFLP profile data sets obtained from the VFAs degradation study indicate that there are no correlations among the data sets from the individual LCFAs. Therefore, these results suggest that the diversity of microorganisms established by each VFA will differ; however, adding LA seems to attenuate these differences. The PCA is consistent with the significant differences detected by the pairwise comparisons between the  $H_2$  yields of the control and LA-treated cultures over 75% of the fermentation period.

## 6.5 CONCLUSIONS

1. LA exerted a strong inhibitory effect on  $H_2$ -consuming microorganisms and reduced their activity by more than 99% when combined with low pH. However, lowering the pH from 7.6 to 5.0 independently from LA-treatment does not strongly inhibit the  $H_2$ -consuming microorganisms.
2. LA increased the abundance of *Clostridium* sp. in glucose-, acetate-, propionate-, and butyrate-fed cultures, but decreased *Clostridium* sp. in  $H_2$ - and lactate-fed cultures; therefore, homoacetogenic species other than *Clostridia* such as

*Moorella thermoacetica*, *Eubacterium*, *Acetobacterium*, and *Syntrophobacter* might play a significant role in H<sub>2</sub> consumption.

3. LA established a heterogeneous spectrum of diverse microbial populations within the active biomass community, and supported physiological conditions favoring the acetate:butyrate type fermentation. Concurrently, it inhibited acetate, propionate, and butyrate degrading microorganisms by 99.9%.
4. Decreasing the pH from 7.6 to 5.0 first and then adding LA increased the biomass concentration of acidogens and acetogens and decreased the biomass concentration of acetate- and H<sub>2</sub>- consumers.
5. LA eliminated homoacetogenic H<sub>2</sub> consumption in H<sub>2</sub>-producing mixed culture; by comparison, increasing homoacetogenic activity was observed in the control culture, which consumed 18% of the maximum H<sub>2</sub> yield.
6. LA-treated H<sub>2</sub>-producing culture was dominated by *Clostridia* (35%); *Bacteroides*, *Flavobacteria*, and *Syntrophus* (28%); *Geobacillus* (10%); *Bacteroides* and *Actinobacillus* (4%); and *Citrobacter*, *Cytophaga*, *Enterobacter*, *Erwinia*, *E. coli*, and *Klebsiella* (3%) while the control culture was dominated by *Bacteroides* sp. (23%); *Bacillus* sp. (19%); *Clostridium* (12%); *Acinetobacter*, and *Falvobacterium* sp. (6%); *Anaeroplasma abactoclasticum* (5%); *Eubacterium* (4.04%); and *Rubrobacter* (3.56%).

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## **CHAPTER 7: EFFECTS OF LAURIC, MYRISTIC, AND PALMATIC ACIDS ON BIOHYDROGEN PRODUCTION IN ANAEROBIC MIXED CULTURE**

### **7.1 INTRODUCTION**

Global hydrogen ( $H_2$ ) consumption amounts to approximately 50 million tonnes of  $H_2$  (worth \$135 billion) per year; and is growing by 10% annually (Winter, 2005). Approximately 96% of the  $H_2$  consumed is produced from non-renewable resources such as fossil fuels by processes that are environmentally problematic and costly (Konieczny *et al.*, 2008). Therefore, environmentally friendly, economical and sustainable methods of  $H_2$  production have to be developed. Hydrogen could be produced biologically as a by-product of the microbial metabolism and according to Zaborsky (1997), this route has been proposed to produce  $H_2$ .

Fermentation depends on the flow of chemical species exchanged among the various trophic groups and their environment and results in the excretion of high energy products (McInnerney and Beaty, 1988). The efflux of the excreted chemical substance could generate a proton-motive force. For example, dark fermentation of sugars by mixed culture consists of the following three syntrophic processes: 1. acidogenesis from sugars to volatile fatty acids (VFAs) and  $H_2$ ; 2. acetogenesis of VFAs to acetate; and 3. methanogenesis from acetate and  $H_2/CO_2$  (Batstone *et al.*, 2002). The entire process could be controlled to recover the energy from  $H_2$  instead of methane.

Theoretically, efficient acidogenic fermentation of glucose would yield 4 mol of  $H_2$  if acetate is the only end product (Eq. 2.1), while only 2 mol of  $H_2$  would be produced if

butyrate is the only end product (Eq. 2.2). Mixed cultures perform a mixed acid fermentation and produce by-products other than acetate and butyrate. Production of propionate (Eq. 2.4) and alcohols (Eq. 7.1) represents a H<sub>2</sub> sink in the dark fermentation route. Moreover, there are other H<sub>2</sub> consuming routes such as homoacetogenic (Eq. 2.14), methanogenic (Eq. 2.15), and sulfidogenic (Eq. 2.20) reactions.



Improving the yield, controlling H<sub>2</sub>-consuming microorganisms and directing excess electron equivalents towards H<sub>2</sub> are basic research objectives in dark H<sub>2</sub> fermentation using mixed cultures. Various inhibitors and stressing agents such as BESA, chloroform, acetylene, heat treatment, and long chain fatty acids (LCFAs) have been used to enhance the H<sub>2</sub> yield. To date, LCFAs are the only renewable biodegradable and non-toxic organic inhibitors that have been used in H<sub>2</sub> production studies. They can be obtained from food processing effluents. LCFAs are known inhibitors to acetoclastic methanogens, and to a lesser extent, to hydrogenotrophic methanogens (Hanaki *et al.*, 1981; Templer *et al.*, 2006). Polyunsaturated LCFAs (C18:1 and C18:2) are known to successfully suppress methane production (Koster and Cramer, 1987; Lalman and Bagley, 2000, 2001), and redirect the flow of electron equivalents towards H<sub>2</sub> (Chowdhury *et al.*, 2007; Ray *et al.*, 2008, 2009). Hydrogen yield of approximately 2.4 mol·H<sub>2</sub> mol<sup>-1</sup> glucose has been reported in studies conducted with LA treated mixed cultures (Ray *et al.*, 2008).

LCFAs shorter than C18, PA, MA, and LUA, result from the anaerobic degradation of C18 and longer chain LCFAs. LUA can exert bactericidal activity against Gram-positive bacteria (Petschow *et al.*, 1996) and is the most inhibitory saturated fatty acid, although less active than the polyunsaturated C18 fatty acids (Ababouch *et al.*, 1994). Inhibition of

acetoclastic methanogens by LUA can begin at 1.6 mM (320 mg L<sup>-1</sup>), and 4.3 mM (860 mg L<sup>-1</sup>) is known to decrease acetoclastic activity by 50% (Koster and Cramer, 1987).

In rumen studies, MA has been reported to act together with LUA and subsequently suppress methanogenic activity. According to Soliva *et al.* (2003), a mixture of 2:1 (C14:C12) was able to suppress methanogenesis by 96% compared to LUA. The ability of shorter chain LCFAs to control the flow of electrons from glucose towards H<sub>2</sub> production is unknown and additional work is required.

During dark fermentation, pH plays an important role in controlling the metabolic pathway (Lay, 2000), and the ultimately affects the yield and the rate of H<sub>2</sub> evolution (Fang and Liu, 2002). An increasing number of studies have indicated that the H<sub>2</sub> yield from dark fermentation favors low pH values (Van Ginkel *et al.*, 2001; Fang and Liu, 2002; Hallenbeck, 2005; Lin and Chang, 1999). This observation was also confirmed in studies conducted with LA and oleic acid (OA) (Ray *et al.*, 2008).

This work examines the effects of LUA, MA, PA and a mixture (50:50) of PA:MA on H<sub>2</sub> production from glucose in anaerobic mixed culture at 37°C with an initial pH 5.0. Attempts to understand the effects of each acid on the H<sub>2</sub> producing microbial community is also part of this study.

## **7.2 MATERIALS AND METHODS**

The experimental design is described in section 3.4.4. The source and maintenance of the inoculum culture are described in section 3.1. The serum batch reactor preparation protocol is given in section 3.2. All analytical methods for liquid and gas products are described in section 3.5. The LCFAs stock solution preparation protocol is described in section 3.5.4.

## 7.3 RESULTS

### 7.3.1 Hydrogen and methane production

Hydrogen was produced and accumulated in all of the cultures incubated under the different conditions examined. After the first glucose injection, the control, LUA, MA, PA, and 50:50 MA:PA treated samples produced maximum  $H_2$  yields of  $2.04 \pm 0.32$ ,  $0.79 \pm 0.10$ ,  $0.97 \pm 0.28$ ,  $2.53 \pm 0.18$ , and  $0.83 \pm 0.71$  mol  $H_2 \cdot mol^{-1}$  glucose, respectively (Table 7.1). After the second glucose injection, the maximum  $H_2$  yield was  $2.24 \pm 0.18$ ,  $1.10 \pm 0.15$ ,  $0.58 \pm 0.14$ ,  $1.99 \pm 0.18$ , and  $1.04 \pm 0.34$  mol  $H_2 \cdot mol^{-1}$  glucose in control, LUA, MA, PA, and 50:50 MA:PA treated samples, respectively.

Table 7.1 Hydrogen yield from glucose in control, LA, MA, PA and MA:PA mixture-treated bottles.

Day	<u><math>H_2</math> yield (mol <math>H_2 \cdot mol^{-1}</math> glucose)</u>				
	<u>Control</u>	<u>Lauric acid</u>	<u>Myristic acid</u>	<u>Palmatic acid</u>	<u>Myristic + Palmatic acid mixture</u>
1	$0.30 \pm 0.23^a$	$0.31 \pm 0.27^a$	$0.21 \pm 0.09^a$	$1.21 \pm 0.16^b$	$0.29 \pm 0.31^a$
2	$1.70 \pm 0.04^a$	$0.73 \pm 0.05^b$	$0.90 \pm 0.10^b$	$1.89 \pm 0.15^a$	$0.54 \pm 0.42^b$
3	<b><math>2.00 \pm 0.32^a</math></b>	<b><math>0.79 \pm 0.10^b</math></b>	$0.89 \pm 0.06^b$	<b><math>2.53 \pm 0.18^a</math></b>	<b><math>0.83 \pm 0.71^a</math></b>
4	$1.77 \pm 0.25^a$	$0.69 \pm 0.04^b$	<b><math>0.97 \pm 0.28^b</math></b>	$1.83 \pm 0.11^a$	$0.50 \pm 0.35^c$
5	$0.61 \pm 0.06^a$	$0.40 \pm 0.12^a$	$0.42 \pm 0.21^a$	$1.84 \pm 0.04^b$	$0.14 \pm 0.08^c$
6	$1.52 \pm 0.15^a$	$0.86 \pm 0.11^a$	$0.57 \pm 0.19^a$	<b><math>1.99 \pm 0.10^b</math></b>	$0.58 \pm 0.48^a$
7	<b><math>2.24 \pm 0.15^a</math></b>	<b><math>1.10 \pm 0.15^a</math></b>	<b><math>0.58 \pm 0.14^b</math></b>	$1.90 \pm 0.14^b$	$0.71 \pm 0.37^a$
8	$1.60 \pm 0.19^a$	$1.05 \pm 0.13^b$	$0.57 \pm 0.10^b$	$1.72 \pm 0.20^a$	<b><math>1.04 \pm 0.34^b</math></b>

Note: <sup>a</sup>, <sup>b</sup> and <sup>c</sup> indicate statistically different means of in the same row. No comparison should be made between rows. Glucose was injected on Day 0 and Day 4. Numbers in bold font indicate the maximum yields after the first and the second glucose injections.

A maximum yield of  $2.53 \pm 0.18$  mol  $H_2 \cdot mol^{-1}$  glucose was measured in the PA-treated samples on day 3 after the first glucose injection. In comparison to other cultures, the PA-treated samples did not show a lag phase in  $H_2$  production after either the first or the



second glucose injections. After the first glucose injection, a 24 h lag phase was observed in the controls, LUA, MA, and MA:PA treated cultures.

Apparent H<sub>2</sub> consumption was observed in the control culture, LUA, PA, and MA:PA-treated cultures after both the first and second glucose injection. The control culture consumed 12 and 29% of the maximum yield (observed on Days 3 and 7) within 24 h. While in the LUA-treated cultures, 12 and 5% of the maximum H<sub>2</sub> yield was consumed after the first and second glucose injections, respectively.

A significant degree of apparent H<sub>2</sub> consumption was observed in PA-treated culture one day after reaching a maximum yield (i.e. on Day 3, three days after the first glucose injection). By Day 4 (four days after the first glucose injection), 28% of the maximum yield produced in PA-treated culture was consumed, whereas four days after the second glucose injection (Day 8), only 14% of the maximum H<sub>2</sub> yield was consumed. Culture treated with a 50:50 PA:MA mixture also showed H<sub>2</sub> consumption after the first glucose injection; 39% of the maximum yield (observed on Day 3) was consumed within 24 h.

The control culture produced methane (CH<sub>4</sub>) to a maximum of 630±125 (Day 4) and 60±20 (Day 8) µmol per bottle four days after the first and second glucose injections. The maximum CH<sub>4</sub> production in cultures treated with LCFAs was 60±33 µmol per bottle; detected in the PA-treated culture on Day 2 (two days after the first glucose injection). Methane was completely inhibited in the LUA-treated culture after the first glucose injection. Overall, CH<sub>4</sub> production decreased to less than 3±1 µmol per bottle in all of the LCFAs-treated cultures after the second glucose injection (Figure 7.1; Days 5 to 8).

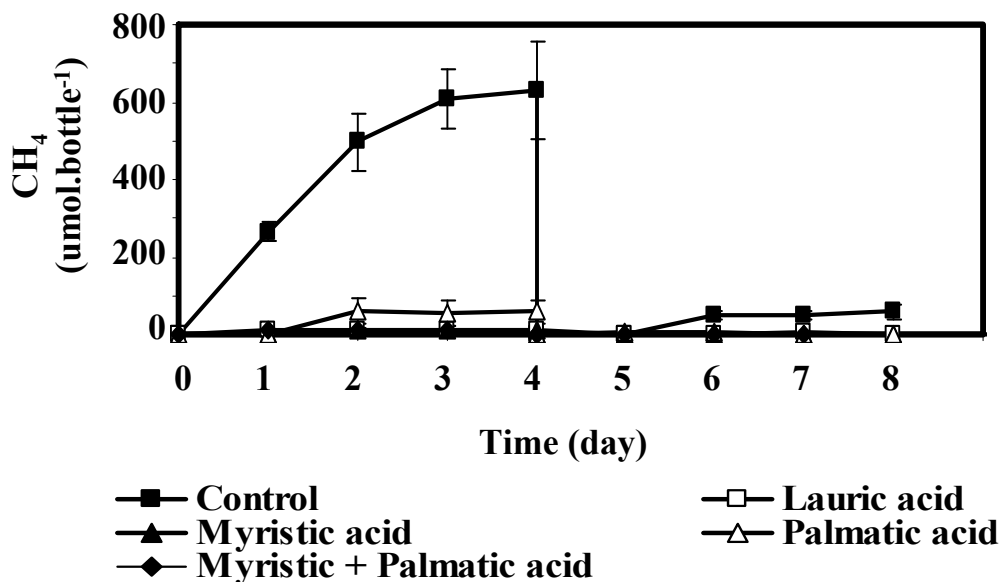


Figure 7.1 Methane production profiles for cultures receiving 7.131 mM LUA, MA, PA and MA:PA plus 5,000 mg L<sup>-1</sup> glucose (Values shown are mean and standard deviation for triplicate samples; Glucose was injected on Day 0 and on Day 4).

### 7.3.2 Glucose degradation

Glucose degradation was inhibited by LUA, MA, PA, and 50:50 MA:PA to different extents. The initial degradation rates for glucose were  $5.5 \pm 0.5$  (control),  $0.7 \pm 0.2$  (LUA),  $1.9 \pm 0.4$  (MA),  $2.5 \pm 0.5$  (PA), and  $1.8 \pm 0.3$  (PA:MA)  $\mu\text{g glucose} \cdot \text{mgVSS}^{-1} \text{ min}^{-1}$ . Based on the controls, the normalized initial glucose degradation rates for LUA, MA and PA treated cultures were 12%, 34%, and 44%, respectively. Bottles treated with a mixture of (50:50) MA:PA had initial glucose degradation rates comparable to that of the MA-treated samples. The rates for the MA and MA:PA -treated samples were  $32 \pm 2\%$  of the initial glucose degradation rate for the controls. The initial glucose degradation rates for the LCFA-treated cultures were significantly less than that of the control culture, but the rates for the MA, PA, and MA:PA treated samples were similar to each other (32 to 44%) (Table 7.2). LUA-treated culture showed the greatest inhibition to the glucose degradation and was significantly different from cultures treated with other LCFAs

(Table 7.2). Generally, 60% of the administered glucose was consumed within 24 hrs in the LCFAs-treated samples while up to 80% was consumed in control samples.

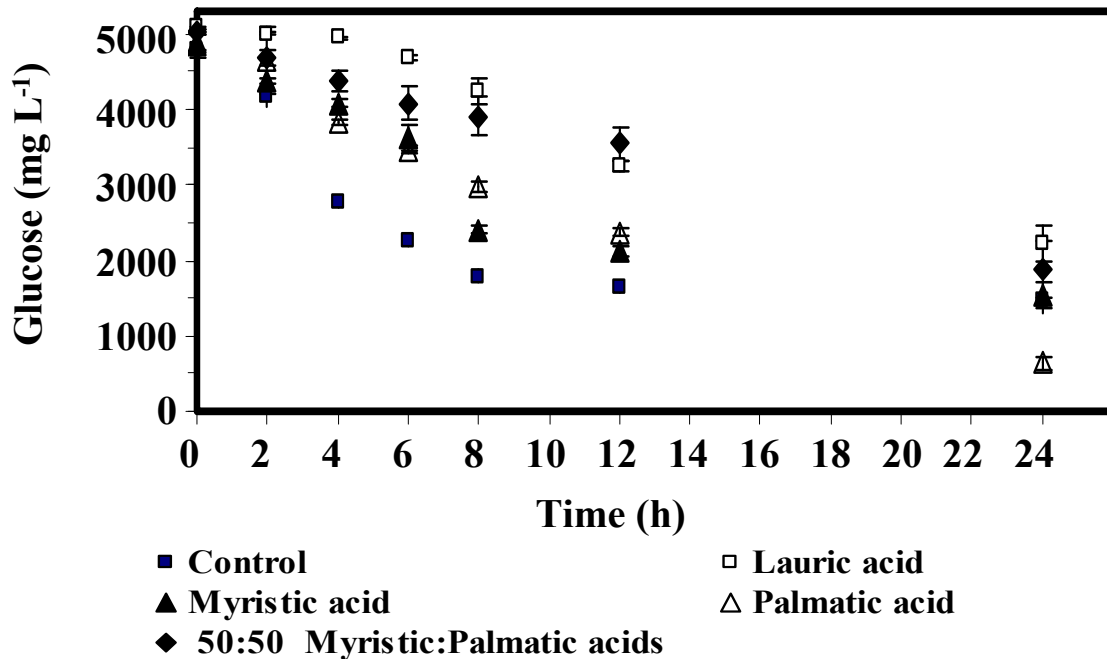


Figure 7.2 Glucose degradation profile over 24 hrs in control and LCFAs- (7.131 mM LUA, MA, and PA) treated cultures fed 5,000 mg L<sup>-1</sup> glucose (Values shown are mean and standard deviation for triplicate samples).

Table 7.2 Initial glucose degradation rate in control, myristic, palmitic and myristic:palmitic acids mixture-treated bottles.

<u>Sample</u>	<u>Initial glucose degradation rate</u> <u>(ug glucose·mg<sup>-1</sup>VSS min<sup>-1</sup>)</u>
Control	5.57±0.48 <sup>a</sup>
LUA	0.67±0.15 <sup>b</sup>
MA	1.88±0.41 <sup>c</sup>
PA	2.46±0.35 <sup>c</sup>
MA + PA	1.78±0.32 <sup>c</sup>

Note: means with similar superscripts are not significantly different.

### 7.3.3 Volatile fatty acids production

The major volatile fatty acids (VFAs) produced were lactate, acetate, propionate and butyrate (Figure 7.3). Lactate was produced in the LUA, MA, PA, and 50:50 MA:PA - treated cultures (Figure 7.3A). MA-treated samples produced up to 730 mg L<sup>-1</sup> of lactate within 4 days following the first glucose injection (Day 4), but accumulated increasing concentrations of up to 3100 mg L<sup>-1</sup> of lactate 4 days after the second glucose injection (Day 8). Samples treated with LUA, PA, and 50:50 MA:PA also produced and accumulated lactate; 4 days after the second glucose injection (Day 8), 760 (LUA), 1200 (PA), and 950 (50:50 MA:PA) mg L<sup>-1</sup> of lactate were detected. PA-treated samples produced lactate only after the second glucose injection (Days 5 to 8). The lactate produced in PA-treated samples was associated with a decrease in H<sub>2</sub> yield after the second glucose injection (Days 5-8) compared to the H<sub>2</sub> yield observed after the first glucose injection (Days 1-4).

Acetate was produced in all cultures under the various LCFA-stress conditions. Control samples produced acetate to a maximum of 1450 mg L<sup>-1</sup> after the first glucose injection, but acetate production increased after the second glucose injection to 2500 mg L<sup>-1</sup> (Figure 7.3B). In cultures treated with LUA, the acetate concentration was 720 mg L<sup>-1</sup> on Day 4 (4 days following the first glucose injection); this level was maintained following the second glucose injection (Days 5-8). Bottles treated with PA produced 1700 (Day 4) and 2400 (Day 8) mg L<sup>-1</sup> acetate 4 days after the first and second glucose injections, respectively. Cultures treated with the 50:50 PA:MA mixture produced less acetate than other samples (525 and 550 mg L<sup>-1</sup> four days after the first and second glucose injections, respectively).

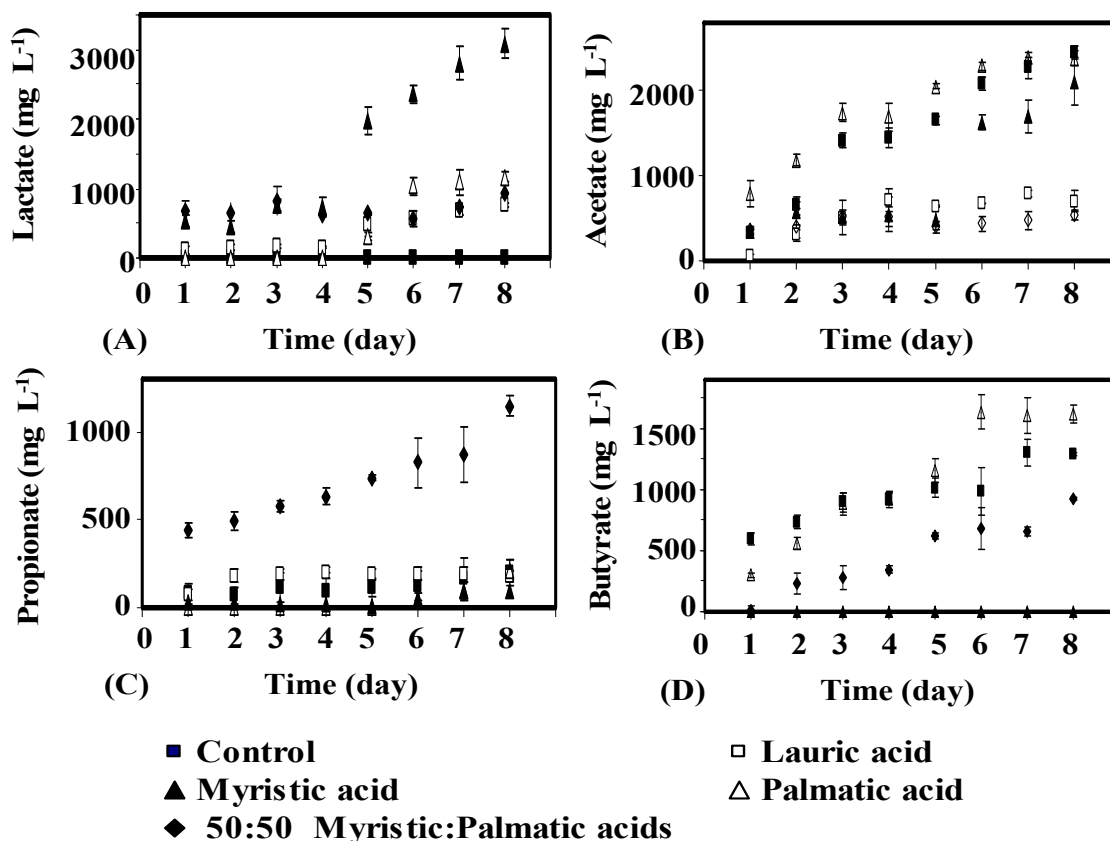


Figure 7.3 VFAs production profiles in control and LCFAs (7.131 mM) -treated cultures fed 5,000 mg L<sup>-1</sup> glucose (Values shown are mean and standard deviation for triplicate samples).

Control samples produced propionate up to 100 mg L<sup>-1</sup> after the first glucose injection (Day 4), and doubled this quantity after the second glucose injection (Figure 7.3C). Increasing propionate concentrations were observed in MA:PA-treated bottles throughout the incubation period; a maximum of 1150 mg L<sup>-1</sup> of propionate was measured four days after the second glucose injection (Day 8). LUA-treated samples produced 180 mg L<sup>-1</sup> on Day 2 after the first glucose injection, and this level was maintained at approximately 190 mg L<sup>-1</sup> until the end of the experiment.

Control and PA-treated samples produced increasing concentrations of butyrate (1020 and 1250 mg L<sup>-1</sup>, respectively) on Day 4 (4 days after the first glucose injection), and

1290 and 1620 mg L<sup>-1</sup> on Day 8 (4 days after the second glucose injection; Figure 7.3D). MA-treated samples did not produce any butyrate, but samples treated with the 50:50 MA:PA mixture produced approximately 350 mg L<sup>-1</sup> (Day 4) and 930 mg L<sup>-1</sup> (Day 8) 4 days after the first and the second glucose injections, respectively.

#### **7.3.4 Alcohols production**

Isopropanol was produced in various quantities in LUA, MA, MA:PA, and PA-treated cultures (Figure 7.4). LUA-treated cultures produced increasing concentrations of isopropanol; a maximum of 1320±150 mg L<sup>-1</sup> was accumulated on Day 8, 4 days after the second glucose injection. The amount of isopropanol produced was LUA > MA > MA:PA > PA. No isopropanol alcohol was produced in the control cultures. Based on this work, it seems that isopropanol production was associated with a decrease in the carbon number of the LCFAs.

Ethanol was produced in all of the cultures in various quantities. LUA-treated culture produced 830±130 mg L<sup>-1</sup> on day 1 after the first glucose injection, and increased up to 900±12 (Day 4) and 1780±70 mg L<sup>-1</sup> (Day 8) 4 days after the first and the second glucose injections, respectively. Cultures treated with MA:PA and PA produced 700±65 and 310±70 mg L<sup>-1</sup>, respectively on Day 8 (4 days after the second glucose injection). PA-treated culture produced more ethanol after the first glucose injection than after the second injection, and MA-treated culture produced a small quantity of ethanol only after the second glucose injection.

Propanol was produced in MA and MA:PA-treated cultures to maximum of 335±70 and 680±40 mg L<sup>-1</sup> respectively on Day 4 (4 days after the first glucose injection). MA:PA-treated cultures accumulated increasing levels of propanol (up to 1034±30 mg L<sup>-1</sup>

<sup>1</sup>) after the second glucose injection. No propanol was observed in the controls, LUA and PA-treated cultures.

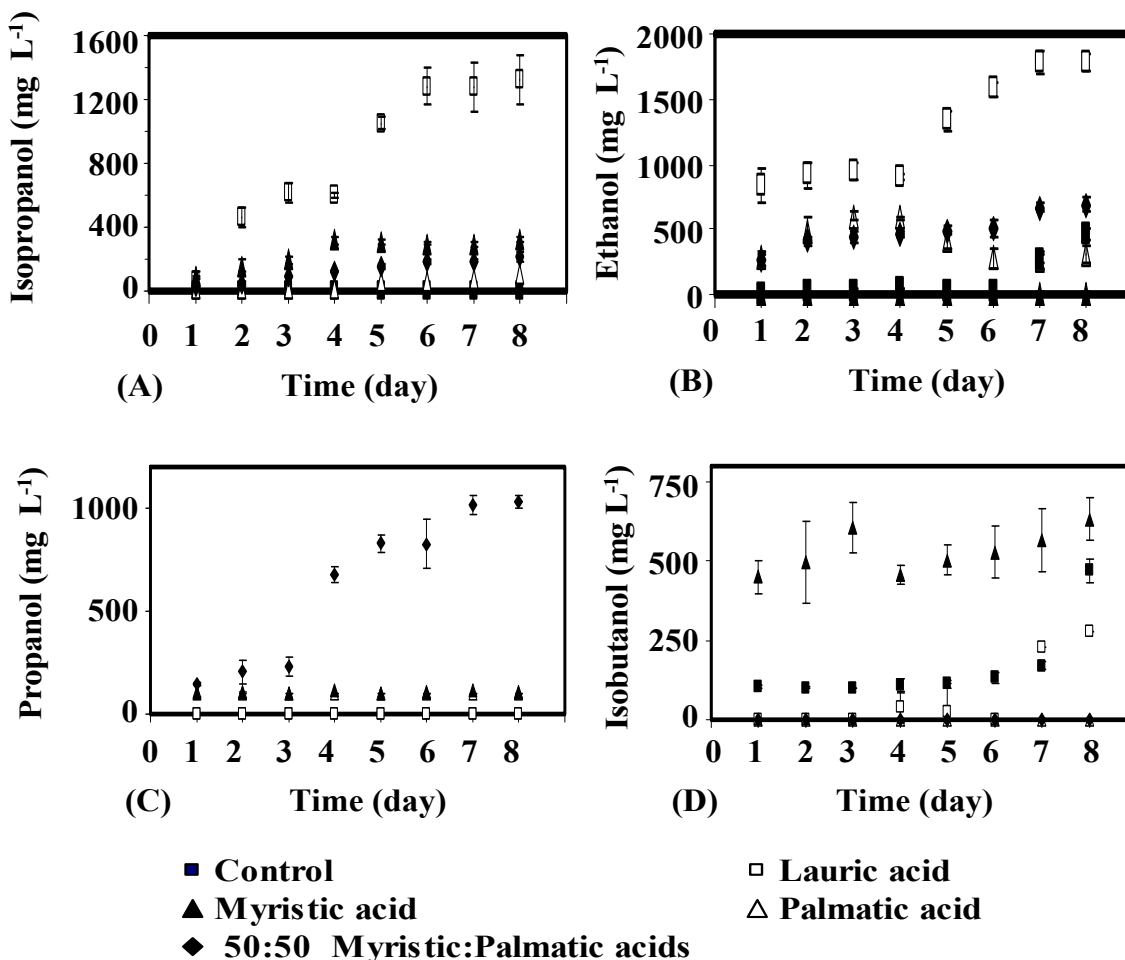


Figure 7.4 Alcohols production profiles in control and LCFAs (7.131 mM) -treated cultures fed 5,000 mg L<sup>-1</sup> glucose (Values shown are mean and standard deviation for triplicate samples).

Isobutanol was produced in the controls, LUA, and MA-treated cultures. Control cultures produced isobutanol beginning on Day 1 (1 day after the first glucose injection) and maintained this level (around 100±10 mg L<sup>-1</sup>) until Day 5 (1 day after the second glucose injection) when the concentration increased successively to a maximum level of 470±40 mg L<sup>-1</sup> on Day 8 (4 days after the second glucose injection). In the LUA-treated cultures, isobutanol was observed on Day 4 (4 days after the first glucose injection), and

the level increased on Day 6 (2 days after the second glucose injection) to a maximum of  $280 \pm 0.0 \text{ mg L}^{-1}$ . MA-treated culture produced increased levels of isobutanol ( $450 \pm 50 \text{ mg L}^{-1}$ ) 1 day after the first glucose injection. Maximum amounts of  $460 \pm 30$  (Day 4) and  $630 \pm 70 \text{ mg L}^{-1}$  (Day 8) of isobutanol were detected 4 days after the first and second glucose injections in MA-treated samples.

### **7.3.5 Electron mass balance**

Electron mass balances are shown in Figure 7.5 for cultures fed with different LCFAS. The procedure used in the electron mass balance and sample calculation are given in Appendix D. The culture treated with PA converted approximately 21% of the electron equivalents available in glucose to  $\text{H}_2$ . This indicates a conversion efficiency of 64% was attained (based on a maximum of 4.0 mol of  $\text{H}_2$  per mol of glucose). Cultures treated with LUA channeled more electron equivalents to alcohols than did the cultures treated with other acids (Figure 7.5D). After the second glucose injection,  $30 \pm 3\%$  and  $35 \pm 1\%$  of the electron equivalents were channeled to isopropanol and ethanol, respectively (Table 7.3).



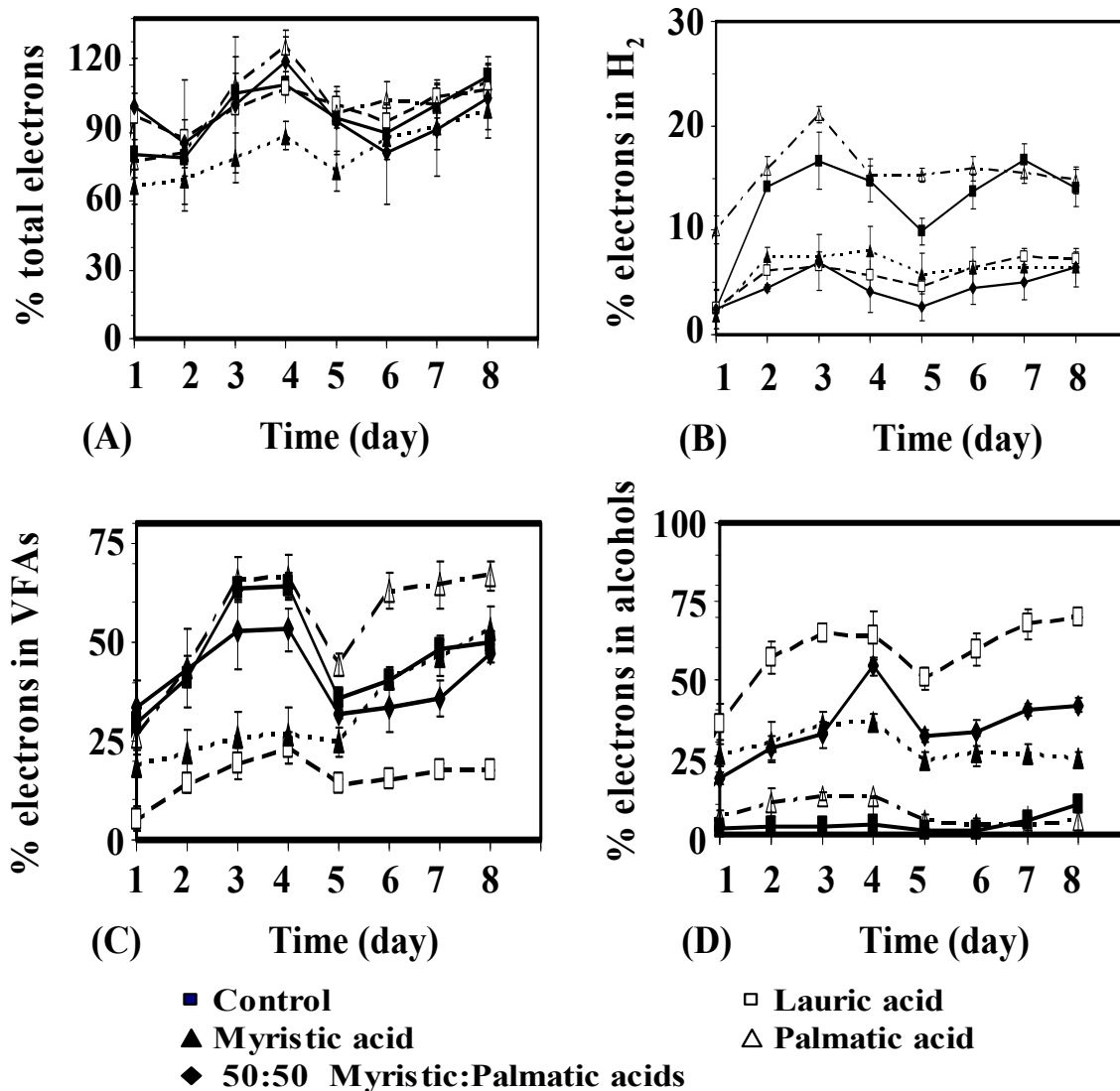


Figure 7.5 Electron mass balance profiles in control and LCFAs (7.131 mM) -treated cultures fed 5,000 mg L<sup>-1</sup> glucose (Values shown are mean and standard deviation for triplicate samples).

Table 7.3 Percent of electron distribution for by-products on the day of maximum H<sub>2</sub> yield after the first and second glucose injections. (Glucose was administered on Days 0 and 4).

Product	Injection	Control	LUA	MA	PA	MA:PA
H <sub>2</sub>	first	16.67±2.67 <sup>a</sup>	6.61±0.37 <sup>b</sup>	8.05±2.31 <sup>b</sup>	21.08±1.49 <sup>a</sup>	6.91±5.91 <sup>b</sup>
	second	18.67±1.25 <sup>a</sup>	7.47±0.71 <sup>b</sup>	6.41±1.56 <sup>b</sup>	15.90±1.20 <sup>a</sup>	6.42±3.02 <sup>b</sup>
CH <sub>4</sub>	first	15.08±0.00	0.22±0.06 <sup>a</sup>	0.23±0.03 <sup>a</sup>	1.34±0.82	0.22±0.03 <sup>a</sup>
	second	8.24±0.00 <sup>a</sup>	0.17±0.03 <sup>b</sup>	0.14±0.01 <sup>b</sup>	0.74±0.40 <sup>b</sup>	0.15±0.00 <sup>b</sup>
HAc	first	29.43±2.24	10.37±2.79 <sup>a</sup>	11.18±4.16 <sup>a</sup>	35.45±2.23	10.46±1.65 <sup>a</sup>
	second	24.99±0.72 <sup>a</sup>	7.16±0.01 <sup>b</sup>	21.36±2.73 <sup>c</sup>	23.29±0.55 <sup>a</sup>	5.50±0.66 <sup>b</sup>
HPro	first	2.55±1.50 <sup>a</sup>	5.30±0.95 <sup>b</sup>	0.69±0.10 <sup>a</sup>	ND <sup>c</sup>	16.30±0.83 <sup>d</sup>
	second	2.84±0.63 <sup>a</sup>	2.69±0.31 <sup>a</sup>	1.36±0.70 <sup>b</sup>	0.76±0.43 <sup>b</sup>	16.27±0.78 <sup>c</sup>
HBut	first	31.82±2.40 <sup>a</sup>	ND <sup>b</sup>	ND <sup>b</sup>	30.48±3.09 <sup>a</sup>	9.83±3.36 <sup>c</sup>
	second	22.24±0.21 <sup>a</sup>	ND <sup>b</sup>	ND <sup>b</sup>	28.24±2.31 <sup>d</sup>	15.99±0.21 <sup>e</sup>
HFor	first	0.10±0.10	ND <sup>a</sup>	ND <sup>a</sup>	0.14±0.08	ND <sup>a</sup>
	second	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	0.37±0.13	ND <sup>a</sup>
HLA	first	0.14±0.00 <sup>a</sup>	3.50±0.37 <sup>a</sup>	14.61±3.02 <sup>b</sup>	0.00±0.00 <sup>a</sup>	16.40±4.15 <sup>b</sup>
	second	0.07±0.00 <sup>a</sup>	6.75±0.35 <sup>b</sup>	30.83±2.12 <sup>a</sup>	10.30±1.36 <sup>b</sup>	9.43±0.76 <sup>c</sup>
Isopropanol	first	ND <sup>a</sup>	27.70±2.89	14.41±0.94	0.29±0.20 <sup>a</sup>	4.33±0.13
	second	ND <sup>a</sup>	28.57±3.37	7.31±0.43 <sup>b</sup>	0.65±0.14 <sup>a</sup>	4.88±0.65 <sup>b</sup>
Ethanol	first	0.74±0.02 <sup>a</sup>	36.97±0.50 <sup>b</sup>	ND <sup>c</sup>	12.04±1.12 <sup>d</sup>	17.42±1.88 <sup>e</sup>
	second	4.68±0.89 <sup>a</sup>	34.84±1.39 <sup>b</sup>	0.19±0.08 <sup>c</sup>	2.78±1.55 <sup>d</sup>	13.44±1.10 <sup>e</sup>
Propanol	first	ND <sup>a</sup>	ND <sup>a</sup>	5.08±0.44 <sup>b</sup>	ND <sup>a</sup>	10.32±2.02 <sup>c</sup>
	second	ND <sup>a</sup>	ND <sup>a</sup>	5.33±0.42 <sup>b</sup>	ND <sup>a</sup>	23.22±0.69 <sup>c</sup>
Isobutanol	first	2.20±0.79 <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>
	second	4.71±0.69 <sup>a</sup>	4.18±0.00 <sup>a</sup>	ND <sup>b</sup>	ND <sup>b</sup>	ND <sup>b</sup>
Butanol	first	ND <sup>a</sup>	ND <sup>a</sup>	16.75±0.00	ND <sup>a</sup>	ND <sup>a</sup>
	second	ND <sup>a</sup>	ND <sup>a</sup>	11.59±0.00	ND <sup>a</sup>	ND <sup>a</sup>

Note: <sup>a</sup>, <sup>b</sup> and <sup>c</sup> indicate significantly different means in the same row. No comparison should be made between rows.

ND = not detected.

### 7.3.6 Principal components analysis

The principal components analysis (PCA) was conducted on the by-products data set for all conditions to detect similarities and differences among the cultures treated with different LCFA. The PCA yielded 3 principal components. The first and second principal components explained 46% and 26% of the variance in the data, respectively. PC1 was correlated with H<sub>2</sub>, acetate, butyrate, and isobutanol whereas PC2 was

correlated with lactate, formate, ethanol, isopropanol, and butanol. PCA indicated differences among the various conditions examined. The responses of the control and PA-treated cultures are close to each other on the visual representation of PC1 and PC2 (Figure 7.6), but the MA- and LUA-treated cultures were far from the control and PA-treated cultures suggesting that their responses were different. The MA:PA treated culture was closer, and thus more similar, to the PA- than the MA-treated culture.

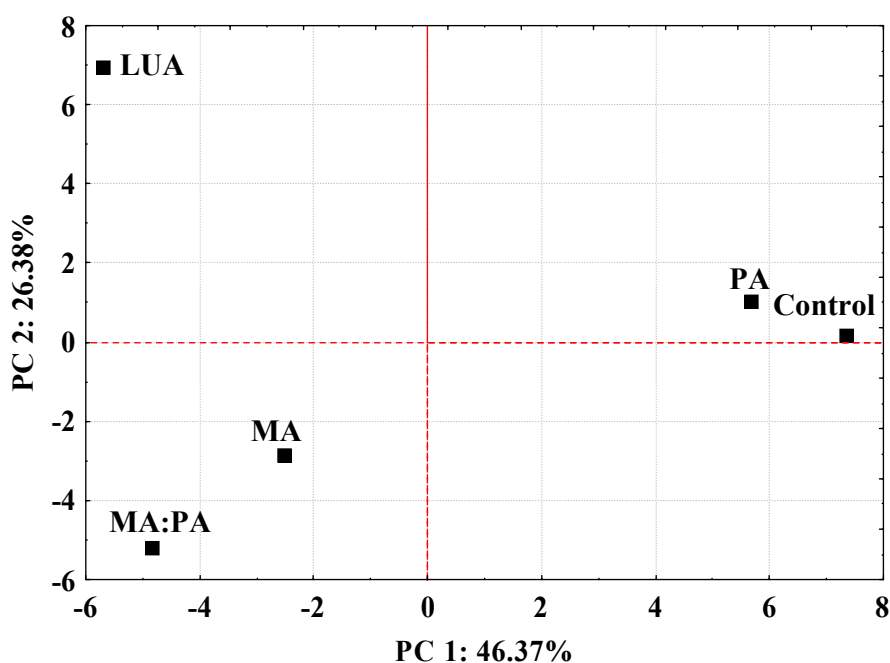


Figure 7.6 Principal component analysis of the by-products data set for control and LUA, MA, PA, and MA:PA -treated samples.

### 7.3.7 Flux Balance Analysis

The purpose the flux balanced analysis (FBA) is to explain the dominant pathway and indicate the  $H_2$  sinks when various LCFAs were added to a mixed culture. The metabolic flux of the culture under the various stress conditions (treatment with LCFAs) was analyzed using MetaFluxNet software, Version 1.8.6.2. The metabolic network of the reactions shown in Figure 3.3 was used to follow the fluxes of the various metabolites

and by-products. The stoichiometries of the reactions (R1 to R30) are provided in Table 3.9. It was assumed that the consortia are capable of metabolizing the various intermediate metabolites and by-products. The fluxes considered included the reactants and products of most commonly reported catabolic bioreactions in glucose fermentation:  $H_2$ , methane, carbon dioxide, lactate, ethanol, butyrate, propionate, acetate that are typically produced in glucose fermentation. Only the NADH/NAD redox-couple is considered for electron transfer. The reaction metabolic network (Figure 3.3) contains 13 intracellular compounds and 15 extracellular compounds that can be exchanged with the environment. Compounds such as pyruvate, NAD, NADH, ACCOA, ACACCOA, and BTCOA exist only inside the cell. The results of the flux balance analysis when maximum  $H_2$  yield was observed for the control, LUA, and MA-treated cultures are given in Figure 7.7(A) and for PA and MA:PA -treated cultures in Figure 7.7(B).

Generally, the ranking of the conditions in terms of total  $H_2$  consumption (mol per mol glucose) was LUA (0.92) > control (0.76) > MA (0.58) > MA:PA (0.43) > PA (0.24). All  $H_2$  consumption in the control (0.76 mol) and PA- treated cultures (0.24 mol) was via acetogenesis (R17 in Figure 7.7) whereas only 55 to 60% of the  $H_2$  consumed in the cultures treated with LUA, MA, or the 50:50 mixture of MA:PA was through acetogenesis.

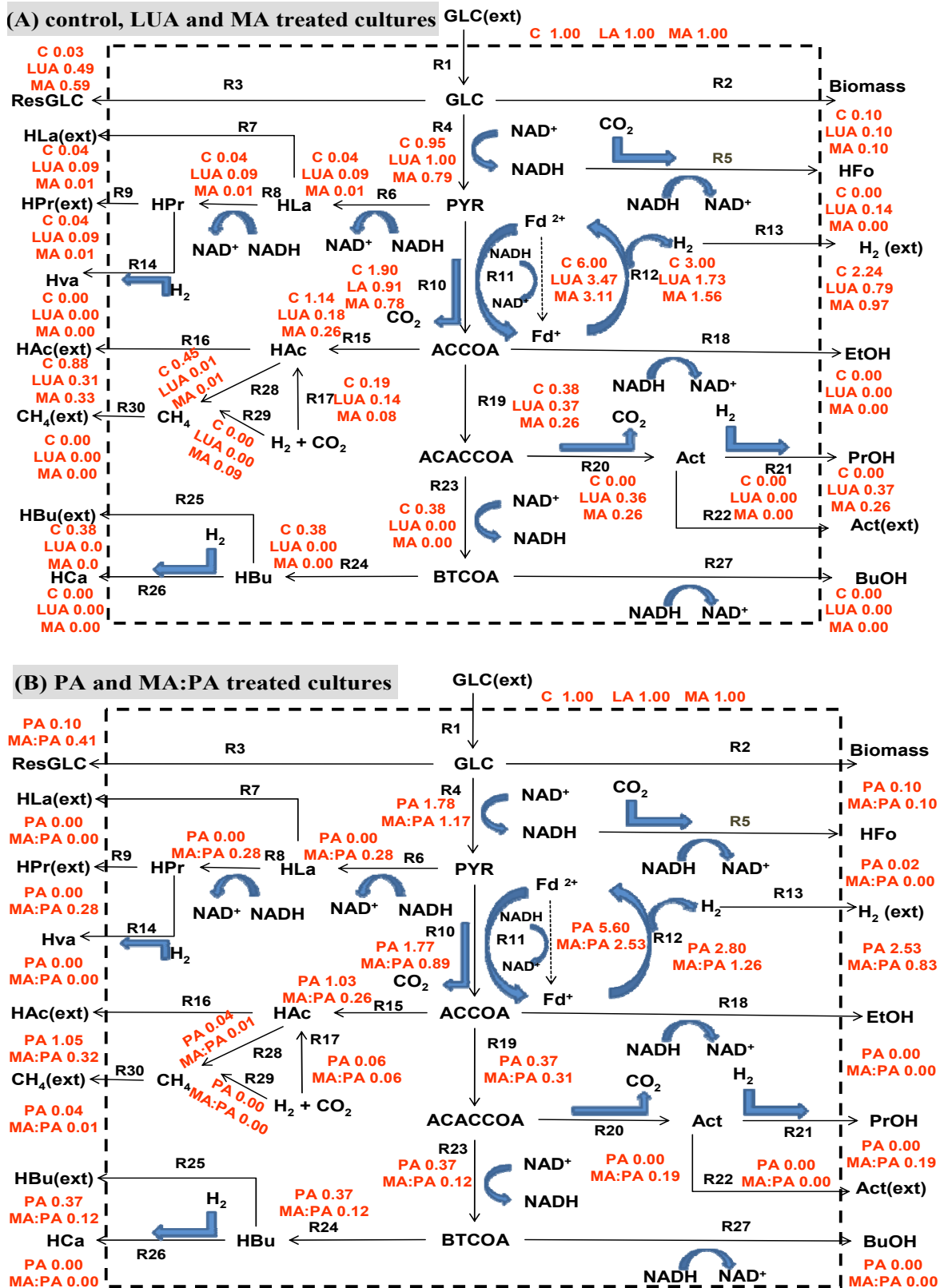


Figure 7.7 Flux balance analysis for maximum H<sub>2</sub> yield in (A) control, LUA, and MA treated cultures, (B) PA and MA:PA treated cultures.

### 7.3.8 Microbiological analysis

Microbial samples from the control culture and the cultures treated with LA, MA, PA, and MA:PA mixtures were analyzed using the T-RFLP method. The band intensity was used to estimate the relative abundance (percentage) of the species represented by each band. The relative abundance was plotted as percentage against the corresponding fragment size (bp) detected for the enzyme *Hae* III in Figure 7.9.

The control culture (lane a in Figure 7.8A and Figure 7.9) was dominated by *Thermoanaerovibrio acidaminovorans* (band 2) 60%; *Eubacterium saphenum*, *Geobacillus* sp., and *Haemophilus* sp. (band 8) 28%; and *Microcystis holsatica* (band 5) 9 %. The relative abundance of *Clostridium* and *Bacillus* (band 9) was less than 1%. However, inoculation with LUA (lane b in Figure 7.8A and Figure 7.9) increased the abundance of spore forming species such as *Clostridium*, *Bacillus*, and *Brevibacillus brevis* (band 8) to 86%. The relative abundance of other species were *Paracoccus dentrifers* (band 1) 5%; *Acidosphaera rubrifacut* (band 1) 3.4%; *Thermoanaerovibrio acidaminovorans* (band 3) 1.4%; and *Eubacterium* (band 6) 1.4%.

The culture treated with MA (lane c in Figure 7.8A and Figure 7.9) was dominated by *Acetobacter* and *Clostridium* sp. (band 8) 67%; *Alkaliphilus metalliredigans* (band 4) 9%; *Capnocytophaga* sp. (band 1) 7 %; *Brevibacillus* sp. (band 8) 4.4%; *Bacteroides*, *Clostridium*, *Bifidobacterium*, and *Flavobacterium* (band 9) 2.6%; *Geobacter* sp. (band 6) 1.5%; and *Thermoanaerovibrio acidaminovorans* (band 3) 1%.

The culture treated with PA (lane d in Figure 7.8A and Figure 7.9) was dominated by *Bacillus* and *Clostridium* (band 6) 34%; *Bacteroides*, *Bacillus*, *Lactobacillus*, and

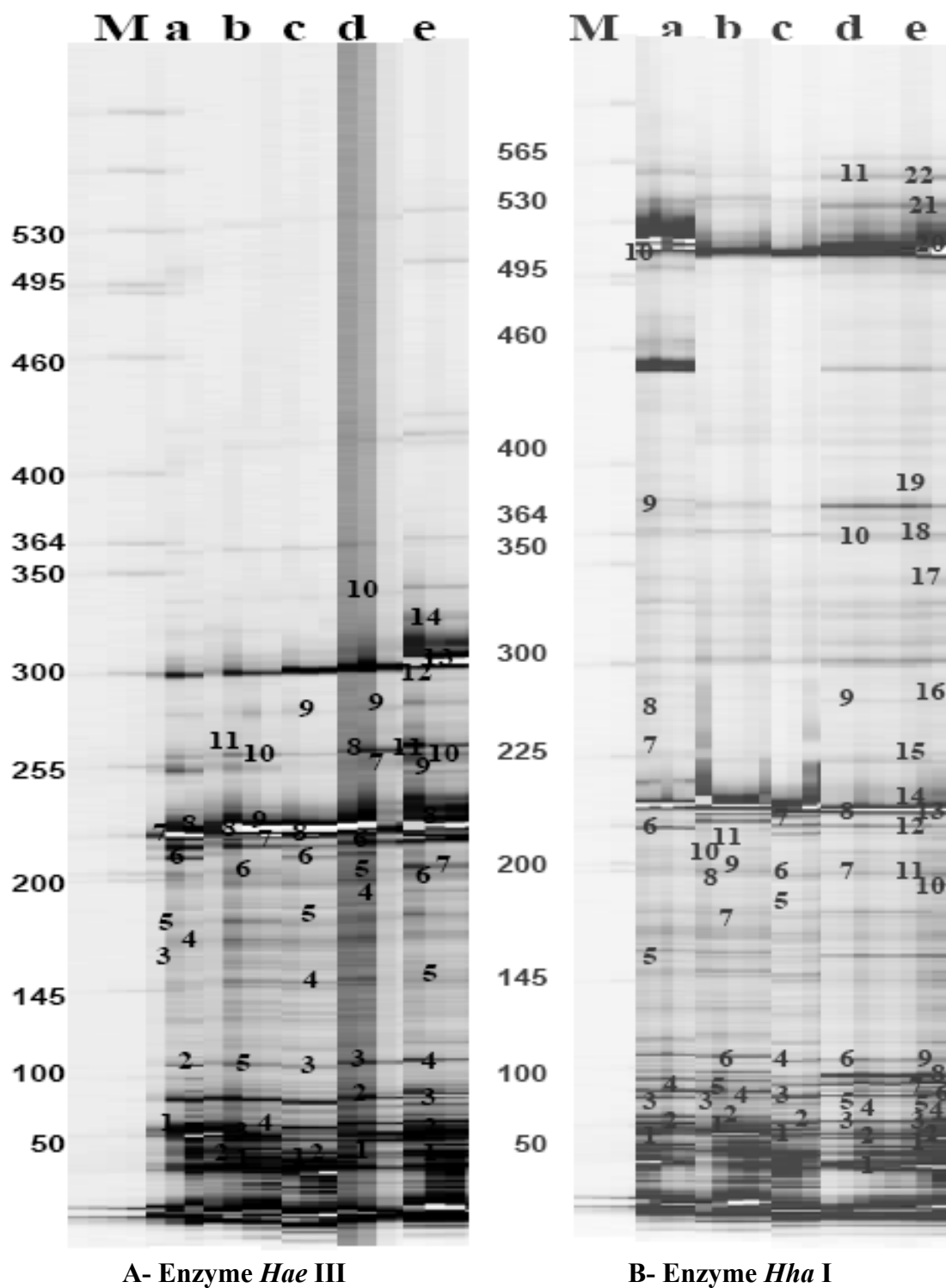


Figure 7.8 Bacterial community profiles in control and cultures treated with LCFAs (LUA, MA, PA, and a mixture of MA:PA). (Lanes a, b, c, d, and e = control, LUA, MA, PA, and MA:PA, respectively). Numbers on lanes indicate the bands identified.

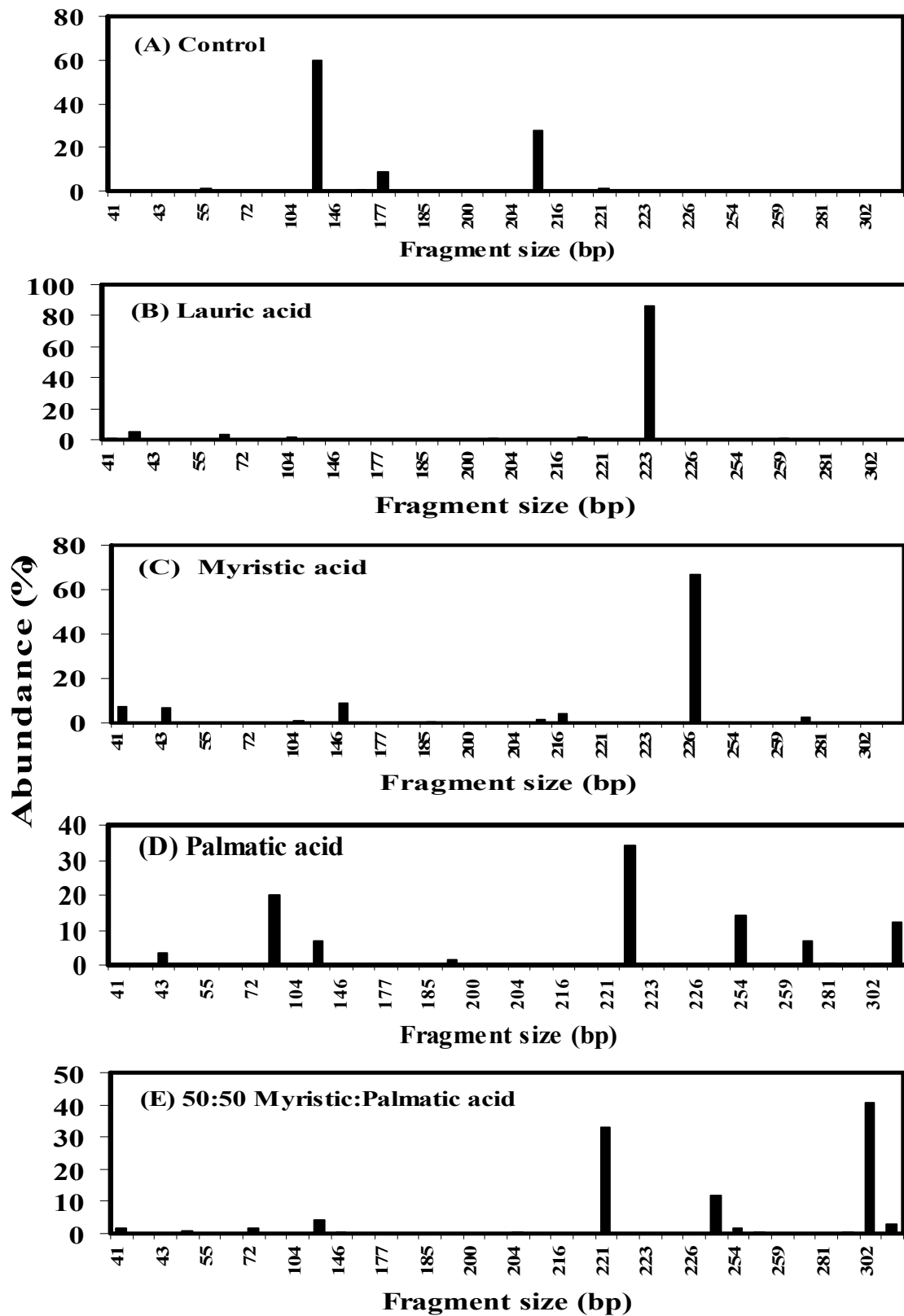


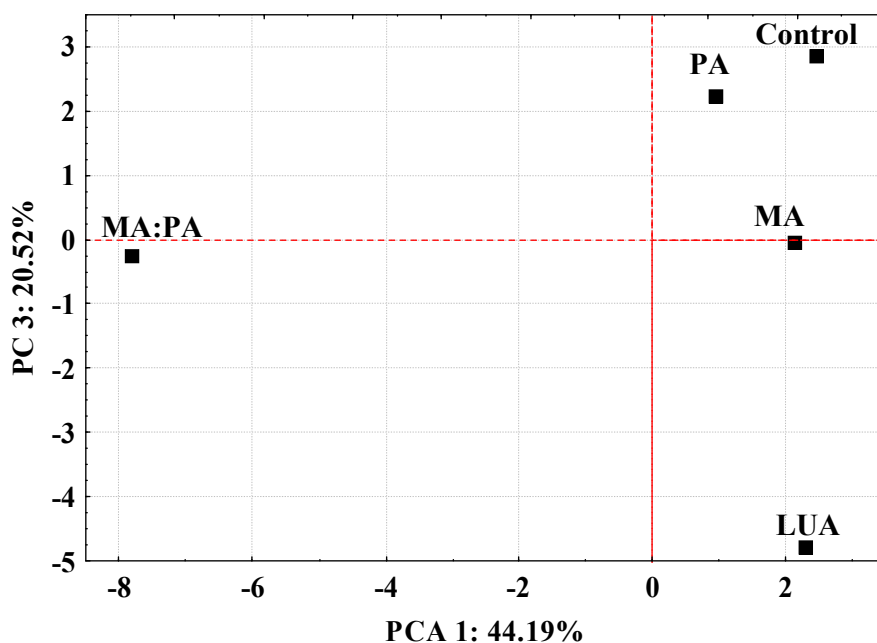
Figure 7.9 Comparison of the terminal fragments detected with the enzyme *Hae* III for control and cultures treated with LUA, MA, PA and MA:PA (LCFAs).



*Bifidobacterium* sp. (band 8) 14%; *Geobacter lovely* (band 10) 12 %; *Bacteroides* (bands 9 and 1) 10%; and *Thermoanaerovibrio acidaminovorans* (band 3) 7%.

The culture treated with a mixture (50:50) of MA:PA (lane e in Figure 7.8A and Figure 7.9) was dominated by *Bacillus* and *Clostridium* (bands 8, 10, and 13) 86%; *Thermoanaerovibrio acidaminovorans* (band 4) 4%; *Butyrivibrio* and *Enterococcus* sp. (band 14) 3%; *Syntrophobacter* sp. (band 3) 1.6%; *Citrobacter*, *Erwinia*, and *Escherichia coli* (band 1) 1.9%.

The PCA of the T-RFLP's profiles yielded 3 PC's which accounted for 88% of the variance in the data set (Figure 7.10). The score map of PC1-PC3 (depicted in the upper panel of Figure 7.10), which accounted for 65% of the variance in the data set, showed the similarity between the control and PA-treated samples.



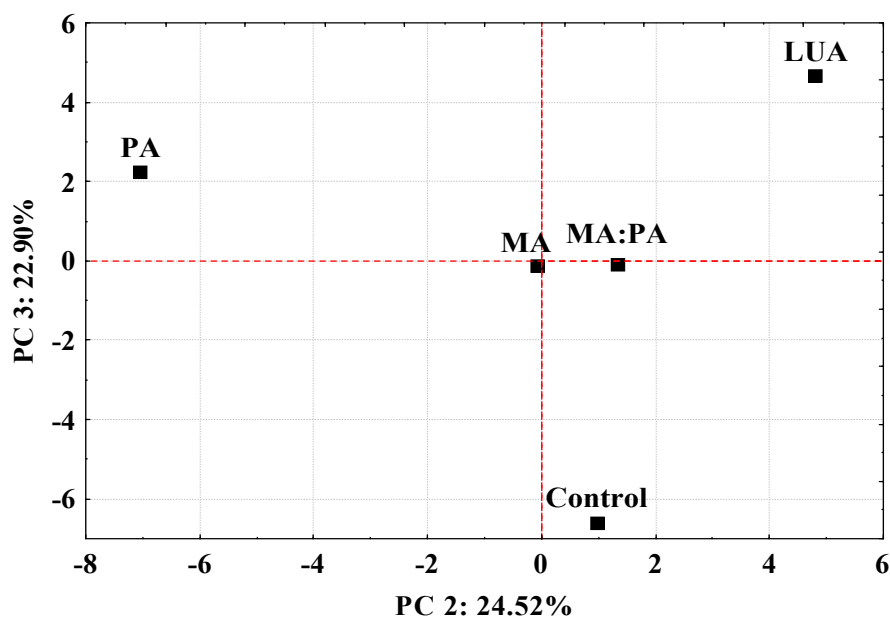


Table 7.10 Score maps of PCA for T-RFLP's profile data set for the control and samples treated with the LCFAs LUA, MA, PA, and MA:PA (50:50).

## 7.4 DISCUSSION

LCFAs are able to impose inhibitory effects on most microorganisms involved in the anaerobic conversion process. Methane-producing Archaeae is the most vulnerable to the inhibition of LCFAs. Recent research into the efficacy of LCFAs as stressing agents in dark biohydrogen production showed promising results (Chowdhry *et al.*, 2007; Ray *et al.*, 2008). To date, only polyunsaturated LCFAs with an 18-carbon chain (LA and OA) have been investigated (Chowdhry *et al.*, 2007; Ray *et al.*, 2008). LCFAs shorter than C18 such as LUA (C12:0), MA (C14:0), and PA (C16:0) also imposes various degrees of inhibition on various anaerobic trophic groups. and in particular methanogens can be severely affected. However, their potential in improving H<sub>2</sub> yield from mixed culture has not been investigated previously.

In this study, anaerobic mixed culture treated with LCFAs shorter than C18 (LUA, MA, PA, or 50:50 MA:PA) produced different quantities of H<sub>2</sub>. These LCFAs affected the H<sub>2</sub> yield, the distribution of products, and the glucose degradation rate to different extents.

The methane profiles in the LCFAs-treated cultures suggest that hydrogenotrophic methanogens were significantly inhibited. Although all of the tested LCFAs inhibited methane production, the H<sub>2</sub> yields varied with the type of LCFA inoculated. Culture treated with PA (C16:0) showed the highest H<sub>2</sub> yield ( $2.53 \pm 0.18$  mol H<sub>2</sub>·mol<sup>-1</sup> glucose) in comparison to cultures treated with LUA (C12:0), MA (C14:0) or a 50:50 mixture of MA:PA (C14:C16). The lowest yield of H<sub>2</sub> was observed in cultures treated with LUA ( $1.10 \pm 0.15$  mol H<sub>2</sub>·mol<sup>-1</sup> glucose) was unexpected because LUA imposes a stronger degree of inhibition on methanogens when compared to the other LCFAs. It is possibly that LUA also inhibited some of the H<sub>2</sub> producers in this study. This is supported by work reported by Soliva *et al.* (2003). They observed H<sub>2</sub> and CH<sub>4</sub> production decreased with increasing C12 concentration. These researchers also found that propionate production consumed some of the H<sub>2</sub> after inhibition of methanogens. In terms of H<sub>2</sub> yield, LUA, MA, and MA:PA-treated cultures were similar to each other, but differed from both the controls and PA-treated cultures. The H<sub>2</sub> yield in the controls and PA-treated cultures were similar. Hence, based on H<sub>2</sub> production, PA-treatment is distinct from cultured treatment with LUA, MA, or MA:PA. This reflects the response of the acidogenic trophic group to this particular LCFA because H<sub>2</sub> production by acidogens is growth-associated. PA seemed to be less harmful to acidogenic H<sub>2</sub> producers in comparison with the other LCFAs. Support for this is indicated by the rate of H<sub>2</sub> production (Table 7.1) and initial

glucose degradation rates (Table 7.2). The extent of the reduction in the glucose-degrading activity due to LUA was 1.5-fold of that due to PA.

Multiple pairwise comparisons of means (using Tukey's test at a 95% confidence interval) indicated that MA, PA and the 50:50 mixture of MA:PA did not differ from each other in terms of the glucose initial degradation rate, but they were different from both the control and LUA-treated cultures. Although the glucose initial degradation rate decreased along with the reduction in carbon number for the tested LCFAs, no conclusion could be drawn to explain this pattern.

Differences in the physical properties of the various LCFAs could contribute to the differences observed in response by the mixed culture under the treatment condition imposed. Inhibition by LCFAs begins with a physical interaction between the LCFA and the cell wall (Maxcy and Dill, 1967). The short-chain LCFAs react in the same manner as the long-chain LCFAs, but the shorter chained acids are less surface-active. Maczulak *et al.* (1981) reported that the inhibitory effects of LCFAs on cell wall digestibility are inversely related to their ability to form insoluble soaps. Thus, the relative inhibitory effects of the different fatty acids may be partially explained by their selective removal from the medium as insoluble soaps. For example, in a mixed culture fermentation, the ranking for completeness of soap formation is stearate > palmitate > oleate (Maczulak *et al.*, 1981). Moreover, Maxcy and Dill (1967) indicated that the adsorption of LCFAs to bacterial surfaces is weak, and the release of biogas desorbs a certain amount of the already adsorbed LCFA (Hwu, 1998). Furthermore, as bacteria enter the exponential growth phase, the ratio of fatty acid to bacterial cells decreases, so LCFAs may have less

of an effect on exponential growth than on growth in the lag phase (Maczulak *et al.*, 1981).

In a methanogenic environment, electron transfer is achieved through the exchange of soluble products and by direct cell-to-cell contact (Stams *et al.*, 2006; Summers *et al.*, 2010). LCFAs form a layer that covers and adheres to the cell wall of methanogens. Therefore, the transport of electrons from one microorganism to another through direct contact is disrupted. This physical separation between the metabolism of the electron donor and the electron acceptor enables energy conservation as  $H_2$  (Stams *et al.*, 2006).

The high yield of  $H_2$  in PA-treated culture was associated with higher percentages of electrons equivalents directed to acetate and butyrate in comparison to the other tested LCFAs (Figure 7.3 and Table 7.3). Conversely, the low yield of  $H_2$  in LUA-treated culture was associated with the production of increasing concentrations of more reduced products such as lactate, isopropanol, and ethanol (Figure 7.3, 7.4, and Table 7.3); this is in agreement with previously published results (Hawkes *et al.*, 2002). The results suggest that PA established the physiological and physico-chemical conditions that directed the bacterial metabolism to the acetate-butyrate fermentation pathway under which the microorganisms produced the maximum  $H_2$  yield.

Under the stress of incubation with LCFAs, the environmental conditions (pH and the hydrogen partial pressure ( $P_{H_2}$ )) change to varying extents, and affected the type and distribution of the by-products. Responses of microorganisms to the new environmental conditions vary from species to species. Thus, the microorganism which catalyzes the thermodynamically most efficient set of reactions under the existing environmental conditions will dominate (Rodríguez *et al.*, 2006). For example, *C. pasteurianum*

produces VFAs and H<sub>2</sub>, but does not grow well at low pH (Nath and Das, 2004). Another example is *Butyribacterium methyilotrophicum*, which produces H<sub>2</sub> during the exponential phase, but then consumes it again during the stationary phase, while acetate and butyrate continue to be produced during the H<sub>2</sub>-consumption period (Annous *et al.*, 1996).

The theoretical maximum H<sub>2</sub> production is associated with a single by-product, i.e., acetate (Eq. 2.1). Practically, as the concentration of acetate increases, the reaction of its production becomes less thermodynamically favorable and active transport of acetate from the intracellular to the extracellular environment consumes more energy (Rodriguez *et al.*, 2006). These conditions force the microorganism to produce other products to reduce the inhibitory effects. Thus, the various LCFAs affected the distribution of by-products (Table 7.3) and environmental conditions to varying degrees. A wider product spectrum was observed in all cultures treated with LUA, MA, PA, and MA:PA. LCFAs shorter than C16 induced shifts to the production of alcohols to varying degrees in comparison to PA. The ranking of the percentages of electron equivalents that were diverted towards alcohol production were LUA (68 % ) > MA:PA (42 % ) > MA (24 % ) > Control (9 % ) > PA (3 % ).

Hydrogen production in the control and PA-treated cultures was associated with acetate-butyrate fermentation. Production of lactate and small quantities of propionate in PA-treated culture after the second glucose injection decreased the H<sub>2</sub> yield. The presence of lactic acid bacteria or their broth supernatant was found to decrease and stop H<sub>2</sub> production by *Clostridia* (Noike *et al.*, 2002). Production of more reduced by-products (lactate and propionate) was due to increased inhibition by PA and the production of VFAs, especially acetate and butyrate.

The distinct effects of LUA- and MA-treatment on the distribution of by-products in comparison to the distribution seen in PA-treated and control cultures are clearly revealed by the PCA of the by-products data set (Figure 7.6). Although the data points for LUA and MA are far from those of the PA-treated and control cultures on the PC1 and PC2 map, the presence of PA in the mixture of 50:50 MA:PA brought the behavior of the culture treated with PA:MA close to that of the control and PA-treated culture. The by-products observed in the LUA- and MA-treated cultures were far from ideal for maximum biohydrogen production. In order to maximize H<sub>2</sub> yield, substrate metabolism should be directed towards VFAs and away from reduced by-products such as lactate and alcohol.

The flux balanced analysis (FBA) (see Figure 7.7) showed that the major H<sub>2</sub> sink in cultures treated with LCFAs was attributed to the homoacetogenic reaction and production of alcohols. Homoacetogenic microorganisms consumed more H<sub>2</sub> in the control and LUA-treated samples in comparison to the MA, PA, and MA:PA-treated samples. Cultures treated with LUA, MA and MA:PA consumed more H<sub>2</sub> in the propanol-producing reaction compared to control and PA-treated cultures (Figure 7.7). The ranking of the cultures according to H<sub>2</sub> consumed (mol of H<sub>2</sub> per mol glucose) by homoacetogenesis was Control (0.19) > LUA (0.15) > MA (0.08) > PA (0.07) > MA:PA (0.06). The ranking based on H<sub>2</sub> consumed by formation of alcohols was LUA (0.37) > MA (0.26) > MA:PA (0.19) > PA (0.004) > Control (0.00). The FBA revealed that the small quantities of methane detected after the first glucose injection was due to the acetoclastic methanogens, which survived the LCFAs inhibition. The concentration of LUA, MA, PA, and the mixture of MA:PA in this study was 7.131 mM. In comparison,

Koster and Cramer (1987) reported that the concentrations of LUA and MA, which resulted in 50% inhibition of acetoclastic methanogens, were 4.3 and 4.8 mM, respectively. The control and LCFA-treated cultures contained various concentrations of *Eubacteria*; some species of these genera are homoacetogens. The relative abundance (percentage) of *Eubacteria* was 28% (control), 1.4% (LUA), 0.2% (MA:PA), and 0.00% (PA) in the control and treated cultures. In addition, the dominant band in MA-treated culture included *Acetobacteria*; members of this species are Gram-positive, non-spore-forming homoacetogenic bacteria (Schink and Bomar, 1991).

PA decreased the growth of the Gram-negative bacteria *Bacteroides ruminicola* and *Butyrivibrio fibrisolvens*, but their growth was stimulated by oleic acid (Maczulak *et al.*, 1981). According to Maczulak *et al.* (1981), PA was much less inhibitory to *Butyrivibrio* sp. than stearic acid. Although *B. fibrisolvens* is classified as Gram-negative, Cheng and Costerton (1977) observed under electron microscopy that it has a Gram-positive-type cell wall. This explains its decreased growth in the presence of PA in comparison with other strictly Gram-negative species. *Butyrivibrio fibrisolvens* was detected in the MA:PA-treated culture with an abundance of approximately 3 %.

The profiles of the T-RFLP analysis revealed that *Thermoanaerovibrio acidaminovorans* dominated the control culture with an abundance of 60% followed by *Geobacillus* and *Eubacterium* (28%), while *Clostridium* sp. formed less than 1% of the biomass. Treating the culture with MA, MA:PA, or LUA increased the abundance of *Clostridium* and *Bacillus* sp. by 67, 75, and 87%, respectively. However, in PA-treated culture the relative abundance of *Clostridium* and *Bacillus* increased to 48% whereas *Thermoanaerovibrio acidaminovorans* decreased to 7%, but the abundance of other



species such as *Geobacter lovely* and *Bacteroides* increased to 12 and 10%, respectively. For example, in the absence of ferric iron, *Geobacter sulfurreducens* can convert acetate to H<sub>2</sub> provided a H<sub>2</sub>-consuming syntrophic partner is present (Cord-Ruwisch *et al.*, 1998). Thus, in this study, *Geobacter* might have converted acetate to H<sub>2</sub> given that solventogenic microorganisms were the H<sub>2</sub>-consuming partners. Generally, PA-treated culture showed a more diverse bacterial population than other cultures treated with LUA, MA, or MA:PA.

Kodick and Worden (1945) reported that LCFAs inhibited *Clostridia*, *Bacillus*, and *Enterobacteriaceae* sp., whereas *Lactobacillus helveticus* (*L. casei* ε) showed growth inhibition to both LA and PA; in comparison, LA did not inhibit *E. coli*.

The maximum yield obtained in the PA-treated culture is comparable to the yield reported for heat-treated inoculum [2.8 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] (van Ginkel and Logan, 2005). This yield is greater than those reported in studies on H<sub>2</sub> production at low pH. Fang and Liu, (2002) reported 2.1 mols H<sub>2</sub>·mol<sup>-1</sup> glucose at pH 5.5 whereas Khanal *et al.* (2004) reported a maximum yield of 1.7 mols H<sub>2</sub>·mol<sup>-1</sup> glucose in a pH range of 4.5 to 6.5. A comparison of the results from this study with previous studies that used LCFAs in H<sub>2</sub> production is shown in Table 7.4. The H<sub>2</sub> yield achieved in the presence of PA is greater than the yield achieved with OA [2.44 and 2.2 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] (Sharma, 2008; Gurukar, 2005) and is comparable or slightly less than that achieved with LA [2.4, 2.7, and 2.8 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] (Ray *et al.*, 2008; Gurukar, 2005; Sharma, 2008).

Table 7.4 Hydrogen yield from glucose fermentation in the presence of LCFAs at 37°C.

<u>LCFA</u>	<u>pH</u>	<u>H<sub>2</sub> yield (mol H<sub>2</sub>·mol<sup>-1</sup> glucose)</u>	<u>Reference</u>
C12	5.0	1.10±0.15	This study
C14	5.0	0.97±0.28	This study
C16	5.0	2.53±0.18	This study
50:50 C14:C16	5.0	1.04±0.34	This study
C18:2	7.6	1.71±0.22	Chowdhry <i>et al.</i> , 2007
C18:2	5.0	2.80±0.19	Sharma, 2008
C18:1	5.0	2.44±0.12	Sharma, 2008
C18:2	5.0	2.4±0.11	Ray <i>et al.</i> , 2008
C18:2	7.6	1.4±0.02	Ray <i>et al.</i> , 2008
C18:2	6.0	2.7±0.06	Grukar, 2005
C18:1	5.0	2.2±0.13	Grukar, 2005

## 7.5 CONCLUSIONS

All the acids examined (LUA, MA, and PA) were effective in suppressing methanogenesis; however, their effects varied in terms of H<sub>2</sub> production. PA was most effective in enhancing the H<sub>2</sub> yield to comparable levels as those obtained using LA. A yield of 2.53 mol H<sub>2</sub>·mol<sup>-1</sup> glucose was obtained from mixed culture treated with PA (7.131 mM). Although PA is less inhibitory than LA, the synergistic effects of the volatile fatty acids produced and PA provided an effective stress condition to direct the electron equivalents towards H<sub>2</sub>. LCFAs shorter than C16 did not produce high H<sub>2</sub> yield. The yield from PA-treated culture is comparable to that obtained from linoleic acid-treated culture.

Treating a mixed culture with MA, 50:50 MA:PA, or LUA increased the percentage of *Clostridium* and *Bacillus* sp. by 67%, 75 %, and 87%, respectively. In LA-treated cultures, the abundance of *Clostridium* and *Bacillus* increased to 48% while the abundance of species such as *Geobacter lovely*, *Bacteroides*, and *Thermoanaerovibrio*

*acidaminovorans* increased to 12%, 10%, and 7%, respectively. PA decreased the quantity of H<sub>2</sub> consumed by producing alcohols and by the homoacetogenic reaction.

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## **CHAPTER 8: LONG-TERM BATCH HYDROGEN PRODUCTION FROM ANAEROBIC MESOPHILIC MIXED CULTURE: EFFECTS OF LINOLEIC ACID DEGRADATION BY-PRODUCTS AND CULTURE STRUCTURE**

### **8.1 INTRODUCTION**

Environmental regulation and protection often competes with policies encouraging economic growth and development. Global economic growth depends upon reliable energy supplies and depleting fossil fuel inventories could impede this growth unless new energy supplies are developed. Green energy sources are under investigation to replace dwindling fossil fuels supplies. Hydrogen (H<sub>2</sub>) production has considerable potential to be an important renewable energy supply. Hydrogen is a clean, efficient, and renewable fuel. Simple biological processes such as dark fermentation, which combines waste treatment with energy generation, can produce H<sub>2</sub> continuously.

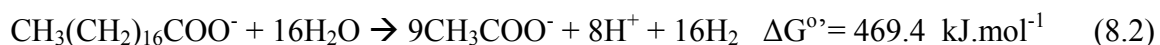
Dark fermentation of a reduced organic substrate by mixed culture produces methane and carbon dioxide through a sequence of interrelated biological reactions mediated by a syntrophy of different anaerobic microbial populations acidogens, acetogens and methanogens. Acidogens and acetogens produce H<sub>2</sub> as an intermediate by-product; however, several microorganisms in the mixed cultures subsequently consume it. Thus, H<sub>2</sub> consumers are responsible for the low H<sub>2</sub> yield in dark fermentation. Research efforts have focused on maximizing H<sub>2</sub> production and minimizing H<sub>2</sub> consumption by microorganisms such as hydrogenotrophic methanogens, homoacetogens, and sulfate-reducing bacteria.

Researchers have investigated various methods to inhibit methanogens and control H<sub>2</sub> consumption in dark fermentation studies. These included heat shock (Logan *et al.*, 2002; Oh *et al.*, 2003; Mohan *et al.*, 2008) or addition of chemical inhibitors such as BES (Mohan *et al.*, 2008; Abreu *et al.*, 2008), chloroform (Abreu *et al.*, 2008), or long chain fatty acids (LCFAs) (Chowdury *et al.*, 2007; Ray *et al.*, 2008, 2010). LCFAs have the edge over other methods because they are environmentally friendly, non-toxic, biodegradable, and natural polymers. Polyunsaturated LCFAs with an 18-carbon chain are known to enhance the H<sub>2</sub> yield from glucose degradation by anaerobic mixed cultures (Chowdury *et al.*, 2007; Ray *et al.*, 2008, 2010). By-products of the degradation of C18 LCFAs can also exert varying degrees of inhibition on the anaerobic consortia (Odongo, 2007; Petschow *et al.*, 1996; Ababouch *et al.*, 1994) and show synergistic effects; for example, lauric acid (LUA) enhanced the toxicity of capric acid (CAA) and myristic acid (MA) (Soliva *et al.*, 2004; Koster and Cramer, 1987).

Degradation of short and long chain fatty acids proceeds through a  $\beta$ -oxidation mechanism (Eq. 8.1), which produces acetate and H<sub>2</sub> as the sub-terminal products before ultimate conversion to methane (Wang and Jeris, 1976). The  $\beta$ -oxidation cycle depends on the activity of methanogens, and the free energy available for the microorganisms controls its progress (Hanaki *et al.*, 1981; Schink, 2002). Accumulation of acetate and H<sub>2</sub> to high levels under inhibited methanogenesis lowers the free energy available for the microorganisms mediating  $\beta$ -oxidation reactions and affects the degradation of the LCFAs themselves (Koster and Cramer, 1987).

Under normal conditions, the  $\beta$ -oxidation process is thermodynamically unfavorable. For example, the free energy change of the individual  $\beta$ -oxidation reactions (Eq. 8.1) by

which linoleic acid (LA, C18:2) is degraded sequentially (to produce one mol of acetate, two mol of H<sub>2</sub>, and one mol of a LCFA with a reduction of two carbons in the alkyl group until the production of C8) is +104.4 kJ mol<sup>-1</sup>. LA is degraded stepwise to palmitic (PA, C16), MA (C14), LUA (C12), capric (CAA, C10) and caprylic acid (CAPA; C8). The free energy of the overall degradation of C18 to acetate and H<sub>2</sub> (Eq. 8.2) is +469.4 kJ.mol<sup>-1</sup>.



The degradation of LCFAs is a slow process especially under the conditions prevalent during H<sub>2</sub> fermentation (Heukeleian and Muller, 1958). In comparison to glucose degradation, the LCFA degradation rate is at least 1500 times less. For instance, the degradation rates for LCFA such as are approximately 0.015-0.34 mg COD g VSS<sup>-1</sup> day<sup>-1</sup> (Alosta, 2002; Hwu, 1997) while rates reaching up to 2.6-23 g COD g VSS<sup>-1</sup> day<sup>-1</sup> have been observed for glucose (Alosta 2002). This is advantageous to the H<sub>2</sub> production process as the inhibitory levels of LCFAs and their by-products are maintained over a relatively long time periods.

The toxicity of LCFAs is correlated with their concentrations (Koster and Cramer, 1987; Angelidaki and Ahring, 1992) and with the culture morphology (Hwu *et al.*, 1996). Cultures with high specific surface area and size distribution are more sensitive to inhibition by LCFAs; flocculent cultures show much greater inhibition than granular cultures (Hwu *et al.*, 1996) because larger microbial surfaces are exposed for adsorption.



High-rate of H<sub>2</sub> production in dark fermentation requires high substrate feeding rates which in turn requires high biomass concentration in a bioreactor. Therefore granulation was suggested as a means to immobilize microorganisms during H<sub>2</sub> production (Chang and Lin., 2004; Lee *et al.*, 2006). However, flocculated and granular cultures (Sayed and Zeeuw, 1988; Sayed *et al.*, 1987) have been used to treat LCFAs-containing wastewater.

Acidogens and acetogens produce H<sub>2</sub> and the latter are able to degrade LCFAs in syntrophy with a H<sub>2</sub>-consuming partner. To date, no published study have compared H<sub>2</sub> production from flocculated and granular cultures under LCFA-inhibitory conditions. Moreover, there are no published reports examining genomic and population shifts during H<sub>2</sub> production under LCFA-inhibition. This study examines H<sub>2</sub> production and LA degradation in granular and flocculated cultures of the same origin at 37°C with an initial pH 5.0 for varying periods of incubation (5 to 30 days). This research relates H<sub>2</sub> production to the incubation period, the by-products of LA degradation, the diversity of the microbial community, and the physical structure of mixed cultures.

## **8.2 MATERIALS AND METHODS**

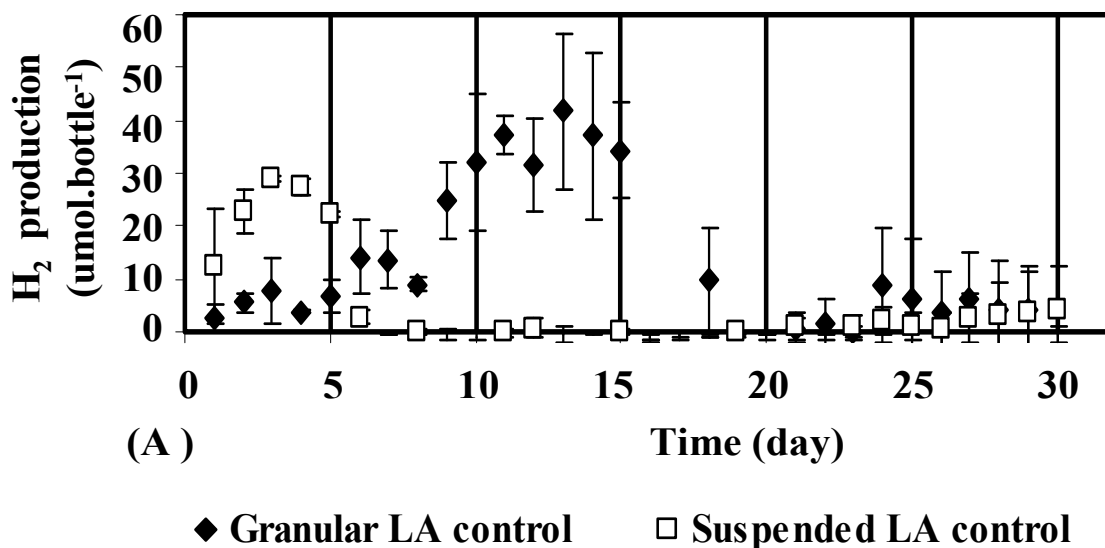
The experimental design is described in section 3.4.5 and is summarized in Table 3.7. Section 3.1 describes the source and maintenance of the inocula. The serum batch reactor preparation protocol is given in section 3.2. The measurement method for LCFAs is described in section 3.5.4 and the optimization of the LCFAs extraction method is described in section 3.5.4.1. All analytical methods for liquid and gas products are described in section 3.5. The protocol for the preparation of the LCFAs stock solution is described in section 3.5.4.

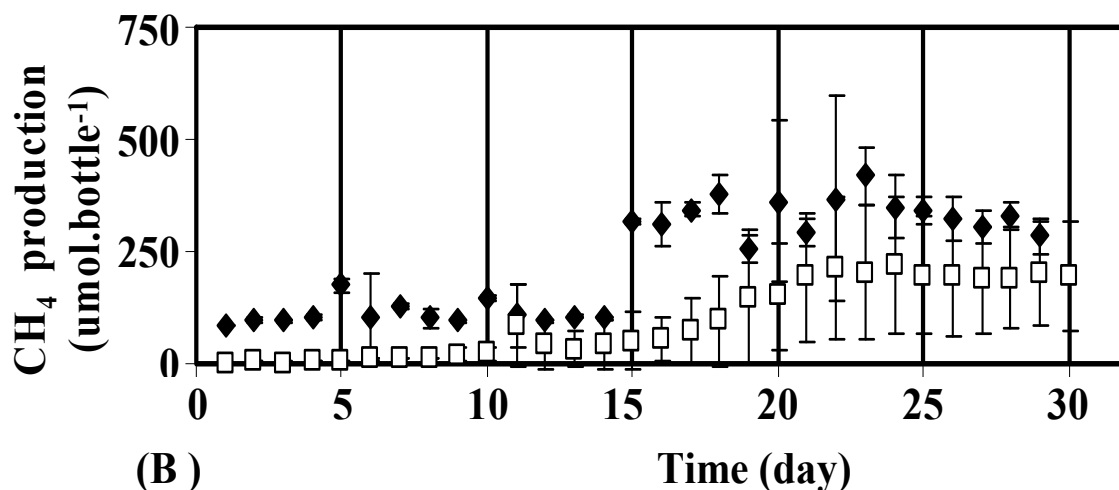
## 8.3 RESULTS

### 8.3.1 LA-treated Control samples

LA-treated control samples (cultures [granular or flocculated] from the same source; incubated with LA for 0 to 30 days) produced small quantities of  $H_2$  and methane ( $CH_4$ ), but the level of production seen throughout the incubation period differed for the granular and flocculated cultures (Figure 8.1). The LA-treated control samples of granular culture (G2) produced a maximum of  $41 \pm 15 \mu\text{mol } H_2$  per bottle on day 13 and  $420 \pm 70 \mu\text{mol } CH_4$  per bottle on day 23. On average, approximately  $100 \mu\text{mol}$  of  $CH_4$  was observed in the LA-treated control samples of the granular culture during the first 15 days of incubation, and approximately  $300 \mu\text{mol}$  of  $CH_4$  during the last 15 days.

The LA-treated control samples of flocculated culture (S2) produced a maximum of  $30 \pm 1 H_2$  per bottle on day 1, which decreased to  $2.8 \pm 1 \mu\text{mol}$  per bottle on day 6 and remained low for the rest of the incubation period. The LA-treated control samples of flocculated culture produced methane at a maximum level of  $220 \pm 150 \mu\text{mol}$  per bottle on day 24.





◆ Granular LA control    □ Flocculated LA control

Figure 8.1 Hydrogen ( $H_2$ ) and methane ( $CH_4$ ) production profiles in LA-treated control samples; granular or flocculated cultures from the same source at varying incubation periods in cultures receiving  $2,000 \text{ mg L}^{-1}$  LA. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

### 8.3.2 Production of VFAs and alcohols in LA-treated control samples

The concentration of acetate increased steadily over the 30-day incubation period in the LA-treated control samples (Figure 8.2A). The acetate concentration peaked between Day 20 and Day 30 at a maximum level of approximately  $750 \text{ mg L}^{-1}$  in the granular culture. A similar trend was observed in the flocculated culture, but with slightly lower concentrations. Propionate was detected at low concentrations in the LA-treated control samples (Figure 8.2B). The concentration of propionate ranged between 100 and  $150 \text{ mg L}^{-1}$  in both flocculated and granular cultures.

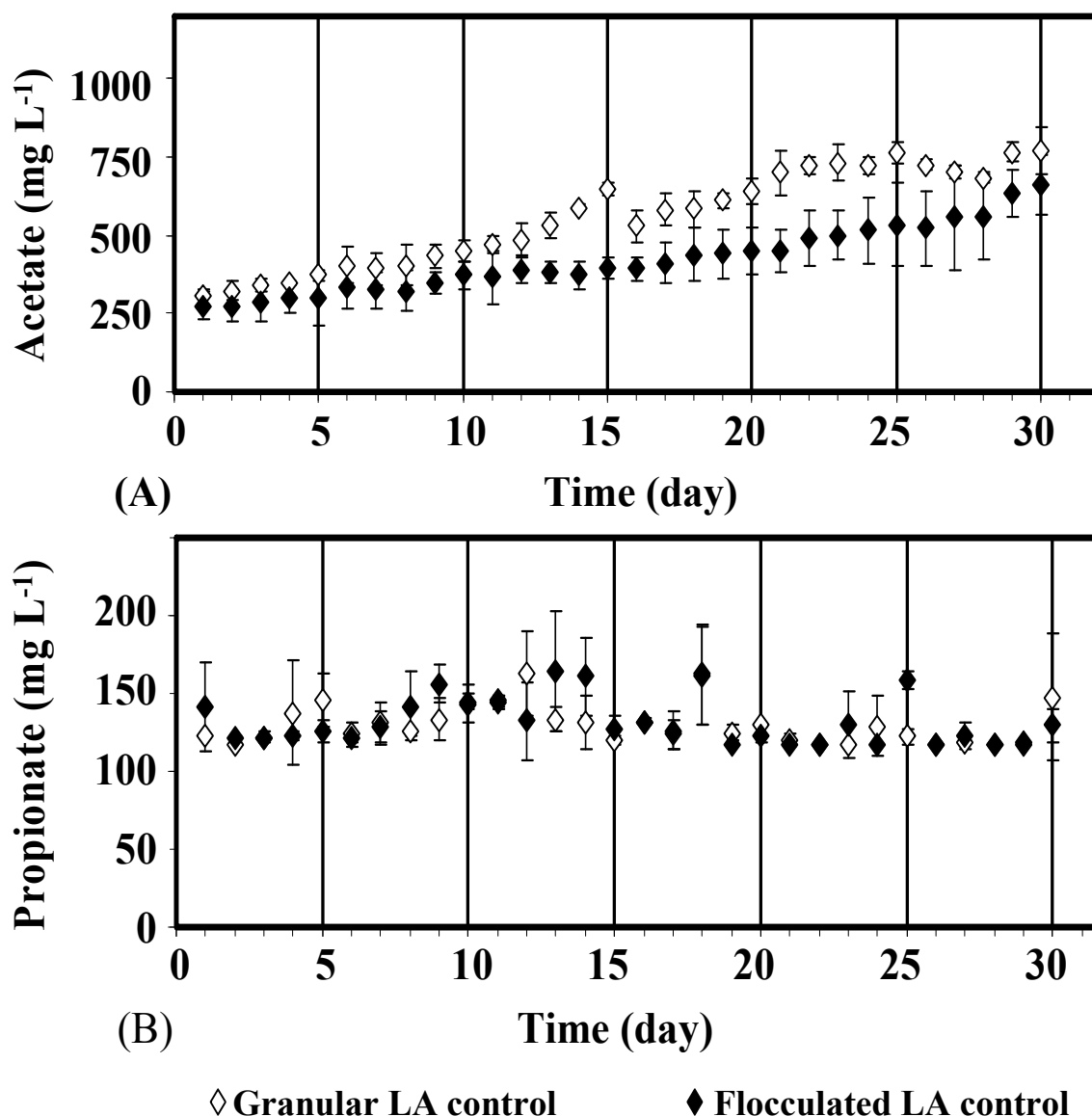


Figure 8.2 Acetate (A) and propionate (B) production profiles in LA-treated control samples at varying incubation periods in granular or flocculated cultures from the same source receiving 2,000 mg L<sup>-1</sup> LA. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

### 8.3.3 Hydrogen and methane production in glucose control cultures

Control cultures (granular or flocculated) were fed glucose, but were not treated with LA (see Section 3.4.5; Table 3.7 for summary of experimental design). Figure 8.3 shows the H<sub>2</sub> and CH<sub>4</sub> production profiles for the glucose controls and glucose-fed plus LA-

treated granular and flocculated cultures over a 30 day period. The results of glucose-fed and LA-treated granular and flocculated cultures will be presented in the next section

8.3.4.

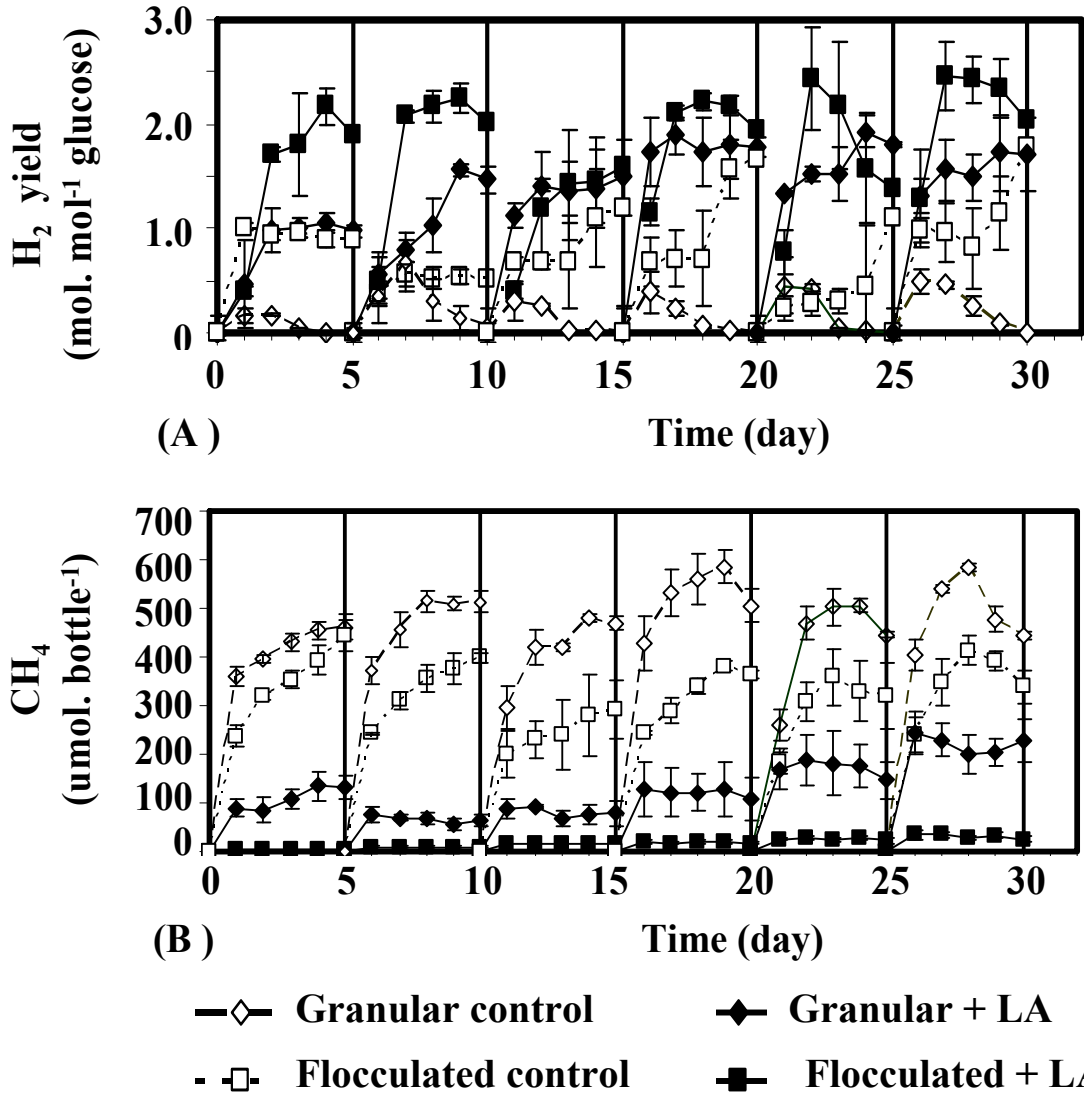


Figure 8.3 Hydrogen and methane production in cultures receiving 2,000 mg L<sup>-1</sup> LA plus 5,000 mg L<sup>-1</sup> glucose; control samples were fed glucose, but were not treated with LA. (A = Hydrogen; B = Methane. Values shown are mean  $\pm$  standard deviation for triplicate samples).

Hydrogen production differed for the granular (G3) and flocculated (S3) glucose control cultures. Notice control samples containing the flocculated culture (S3) produced

more  $H_2$  than control granular cultures (G3). Furthermore, control samples from the granular culture (G3) showed significant  $H_2$  consumption 1 day after each glucose injection throughout the experiment.  $H_2$  was not detected 5 days after each glucose injection. Table 8.1 shows the maximum  $H_2$  yield in glucose control samples (G3 and S3) for different incubation periods. Maximum levels of  $H_2$  were  $0.68 \pm 0.28 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose after 5 days of incubation and  $1.79 \pm 0.35 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose after 25 days of incubation in glucose control samples of granular and flocculated cultures, respectively (Table 8.1).

Both glucose control cultures (G3 and S3) produced  $CH_4$  throughout the experiment. The average quantity of  $CH_4$  produced for different incubation periods are shown in Table 8.2. Generally, control samples of granular culture (G3) produced more  $CH_4$  than control samples containing flocculated culture (S3).

Table 8.1 Maximum  $H_2$  yield in untreated glucose control and LA-treated granular and flocculated cultures incubated for 0, 5, 10, 15, 20, 25, and 30 days. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

Incubation period (day)	<b><math>H_2</math> yield (<math>\text{mol } H_2 \cdot \text{mol}^{-1}</math> glucose)</b>			
	<b><u>Granular culture (G)</u></b>		<b><u>Suspended culture (S)</u></b>	
	<b>Glucose control</b>	<b>Glucose + 2,000 (<math>\text{mg L}^{-1}</math>) LA</b>	<b>Glucose control</b>	<b>Glucose + 2,000 (<math>\text{mg L}^{-1}</math>) LA</b>
	<b><u>(G3)</u></b>	<b><u>(G1)</u></b>	<b><u>(S3)</u></b>	<b><u>(S1)</u></b>
0-5	$0.18 \pm 0.07^a$	$1.06 \pm 0.08^b$	$1.02 \pm 0.03^b$	$2.17 \pm 0.49^c$
5-10	<b><math>0.68 \pm 0.28^a</math></b>	$1.57 \pm 0.06^b$	$0.56 \pm 0.10^a$	$2.25 \pm 0.15^c$
10-15	$0.31 \pm 0.25^a$	$1.49 \pm 0.36^b$	$1.20 \pm 0.48^b$	$1.60 \pm 0.41^b$
15-20	$0.39 \pm 0.24^a$	$1.89 \pm 0.17^b$	$1.66 \pm 0.28^b$	$2.22 \pm 0.08^c$
20-25	$0.45 \pm 0.08^a$	<b><math>1.93 \pm 0.15^b</math></b>	$1.09 \pm 0.58^a$	$2.44 \pm 0.20^c$
25-30	$0.49 \pm 0.08^a$	$1.71 \pm 0.35^b$	<b><math>1.79 \pm 0.35^b</math></b>	<b><math>2.52 \pm 0.20^c</math></b>

Note: <sup>a, b</sup> and <sup>c</sup> indicate significantly different means in the same row.

Table 8.2 Average methane production in untreated glucose control and LA-treated granular and flocculated cultures incubated for 0, 5, 10, 15, 20, 25, and 30 days. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

Incubation period (day)	Methane production ( $\mu\text{mol CH}_4/\text{bottle}$ )			
	Granular culture (G)		Suspended culture (S)	
	Glucose control	Glucose + 2,000 ( $\text{mg L}^{-1}$ ) LA	Glucose control	Glucose + 2,000 ( $\text{mg L}^{-1}$ ) LA
	(G3)	(G1)	(S3)	(S1)
0-5	420 $\pm$ 42 <sup>a</sup>	110 $\pm$ 23 <sup>b</sup>	349 $\pm$ 78 <sup>a</sup>	3.0 $\pm$ 0.9 <sup>c</sup>
5-10	473 $\pm$ 62 <sup>a</sup>	67 $\pm$ 7 <sup>b</sup>	337 $\pm$ 62 <sup>c</sup>	8.8 $\pm$ 1.3 <sup>d</sup>
10-15	417 $\pm$ 73 <sup>a</sup>	80 $\pm$ 9 <sup>b</sup>	249 $\pm$ 38 <sup>c</sup>	16 $\pm$ 1 <sup>d</sup>
15-20	523 $\pm$ 61 <sup>a</sup>	121 $\pm$ 7 <sup>b</sup>	324 $\pm$ 56 <sup>c</sup>	19 $\pm$ 1 <sup>d</sup>
20-25	437 $\pm$ 101 <sup>a</sup>	172 $\pm$ 16 <sup>b</sup>	300 $\pm$ 67 <sup>a</sup>	25 $\pm$ 2 <sup>c</sup>
25-30	490 $\pm$ 72 <sup>a</sup>	221 $\pm$ 19 <sup>b</sup>	346 $\pm$ 67 <sup>c</sup>	31 $\pm$ 4 <sup>d</sup>

Note: <sup>a</sup> and <sup>b</sup> indicate statistically similar means in the same row; otherwise, any two means in the same row are significantly different.

#### 8.3.4 Hydrogen and methane production in granular or suspended cultures fed glucose and treated with LA

The effects of varying incubation periods from 0 to 25 days and the by-products of LA degradation on  $\text{H}_2$  and methane production from glucose in granular and flocculated mixed culture were assessed. The effect of varying incubation periods on  $\text{H}_2$  yield and  $\text{CH}_4$  production is shown in Figure 8.4. Hydrogen was produced in the cultures fed glucose and treated with LA (G1 and S1) to varying degrees over the incubation periods. A slight lag in  $\text{H}_2$  production was observed in LA-treated cultures (G1 and S1) incubated for 0 to 10 days, but was not observed for cultures incubated from 15 to 25 days. The maximum  $\text{H}_2$  yield for the various incubation periods is given in Table 8.1. Hydrogen levels peaked within 1 to 2 days after glucose injection in LA-treated cultures of granular and flocculated culture (G1 and S1) incubated for various periods.

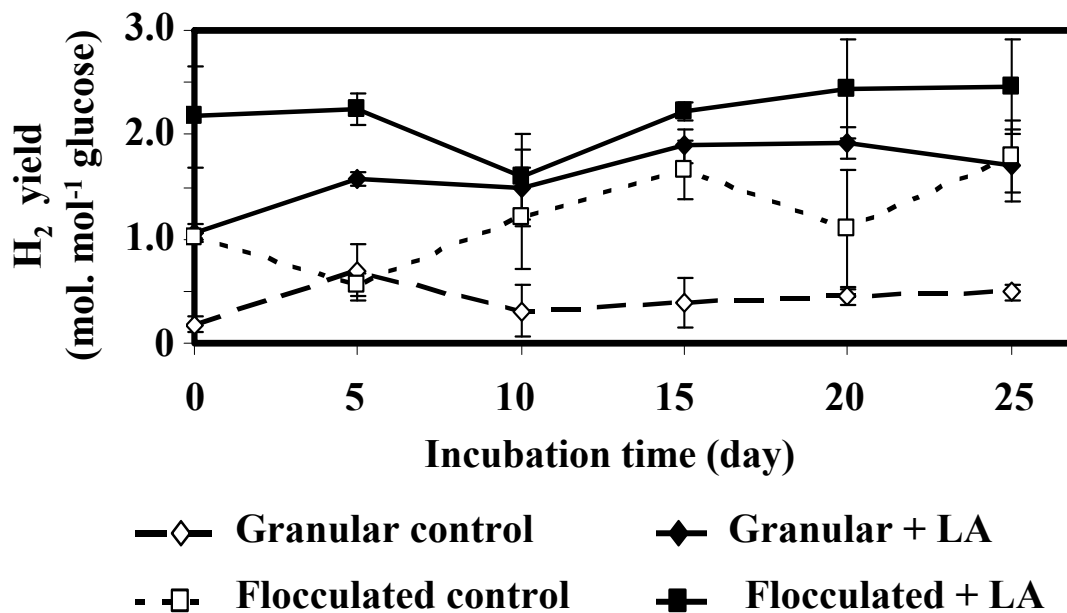


Figure 8.4 Maximum yield of H<sub>2</sub> in glucose control and glucose plus LA-treated samples (2,000 mg L<sup>-1</sup>) from suspended and granular culture incubated for 0, 5, 10, 15, 20, 25, and 30 days. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

Generally, flocculated culture treated with LA (S1) produced more H<sub>2</sub> than granular culture (G1) during the incubation period. The maximum H<sub>2</sub> yields measured in LA-treated flocculated (S1) and granular (G1) cultures were  $2.5 \pm 0.2$  and  $1.9 \pm 0.2$  mol H<sub>2</sub>·mol<sup>-1</sup> glucose after incubation periods of 25 and 20 days, respectively. Varying the incubation time did not result in any particular trend in the H<sub>2</sub> yield (Figure 8.3). The smallest H<sub>2</sub> yields in LA-treated cultures were  $1.1 \pm 0.1$  and  $1.6 \pm 0.4$  mol H<sub>2</sub>·mol<sup>-1</sup> glucose, observed in granular and flocculated culture (G1 and S1; incubated for 0 and 10 days, respectively).

Pairwise comparisons (Tukey's test; 95.0% confidence levels) identified differences in maximum H<sub>2</sub> yield amongst the cultures over the various incubation periods (Table 8.1). Only two of the pairs of LA-treated samples of granular and flocculated cultures (those incubated for 15 and 20 days) were similar. Hydrogen yields in glucose-control samples of the granular culture (G3) were significantly different from their corresponding yields



in LA-treated samples (G1) for all incubation periods. Only three pairs of the control- and LA-treated samples of the flocculated culture (S3 and S1 incubated for 5, 10, and 15 days) produced similar H<sub>2</sub> yields.

Varying degrees of H<sub>2</sub> consumption were observed in LA-treated cultures. Flocculated culture treated with LA (S1) showed increased H<sub>2</sub> consumption after 20 and 25 days of incubation in comparison to other incubation periods. The quantity of H<sub>2</sub> that remained in flocculated culture 5 days after injecting glucose was 56% and 81% of the maximum produced in cultures incubated for 20 and 25 days, respectively. Generally, 80% of the peak H<sub>2</sub> levels produced by S1 remained for 5 days after injecting glucose regardless of the incubation period. The exception was S1 samples which were incubated for 20 days.

Glucose-control granular cultures (G3) consumed H<sub>2</sub> rapidly with almost 99% of the maximum H<sub>2</sub> produced consumed within 5 days of after injecting glucose over the incubation period. Glucose-control flocculated culture (S3) incubated for 10 days or more; however, were characterized by a relatively stable H<sub>2</sub> production phase for almost 3 days following glucose injection. Increasing production was observed on days 4 and 5 (Figure 8.3).

Both control and glucose plus-LA -treated cultures produced CH<sub>4</sub>. Granular culture produced increasing quantities of CH<sub>4</sub> throughout the incubation period. Although the quantity of CH<sub>4</sub> produced in LA-treated granular and flocculated cultures (G1 and S1) increased with the length of incubation (Table 8.2), less than 220 and 30  $\mu$ mol of CH<sub>4</sub> per bottle were observed in these LA-treated samples. In contrast, maximum levels of  $490 \pm 72$  and  $346 \pm 67$   $\mu$ mol of CH<sub>4</sub> per bottle were observed in the corresponding glucose-controls (G3 and S3).

### 8.3.5 Glucose degradation

The profiles of glucose degradation in glucose-control (G3 and S3) and LA-treated samples of granular and flocculated cultures (G1 and S1) are shown in Figure 8.5. LA affected the initial degradation rate of glucose in the flocculated culture more than in granular culture. The initial degradation rate in flocculated culture decreased by approximately 50% (from  $1.9 \pm 0.4 \text{ mg L}^{-1}$  in glucose control samples (S3) to  $0.9 \pm 0.2 \text{ mg L}^{-1}$  in samples fed glucose and treated with LA (S1)). In comparison, in the granular culture, the rate decreased by 24% (from  $2.7 \pm 0.9 \text{ mg L}^{-1}$  in glucose control samples (G3) to  $2.1 \pm 0.8 \text{ mg L}^{-1}$  in samples fed glucose and treated with LA (G1)). Given that both

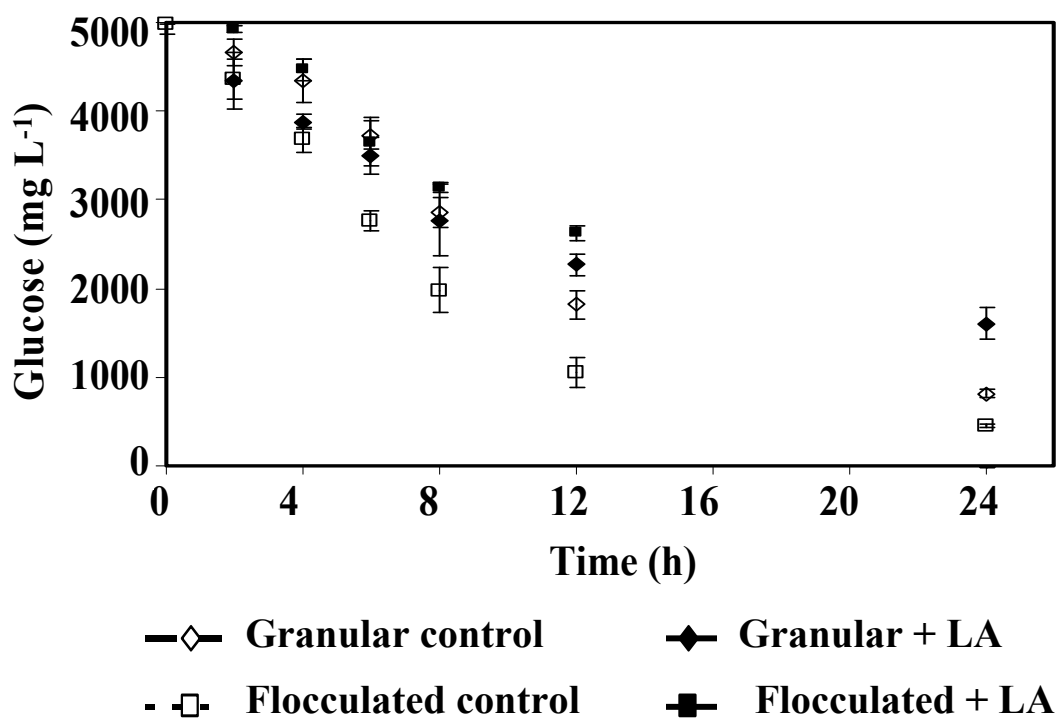


Figure 8.5 Glucose degradation profiles in glucose-control and glucose plus LA-treated ( $2,000 \text{ mg L}^{-1}$ ) samples from flocculated and granular cultures. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

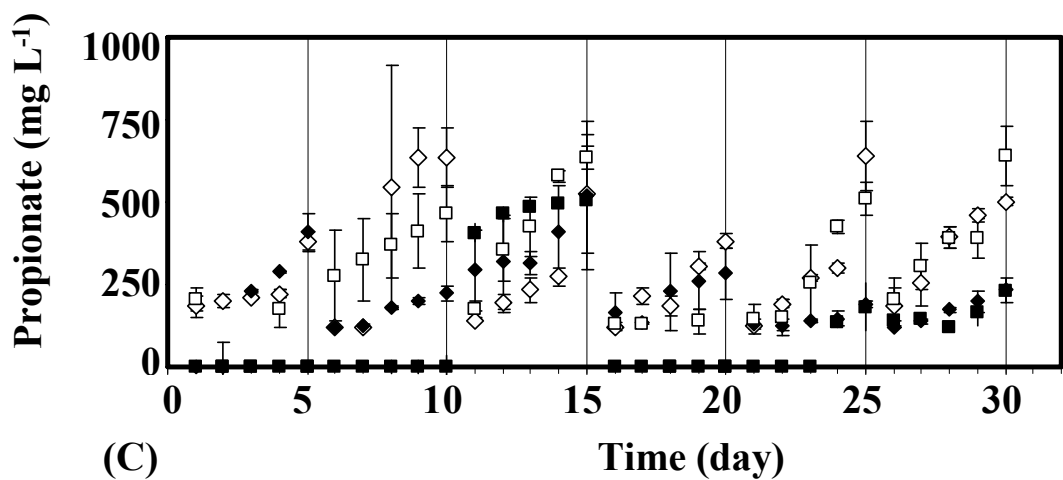
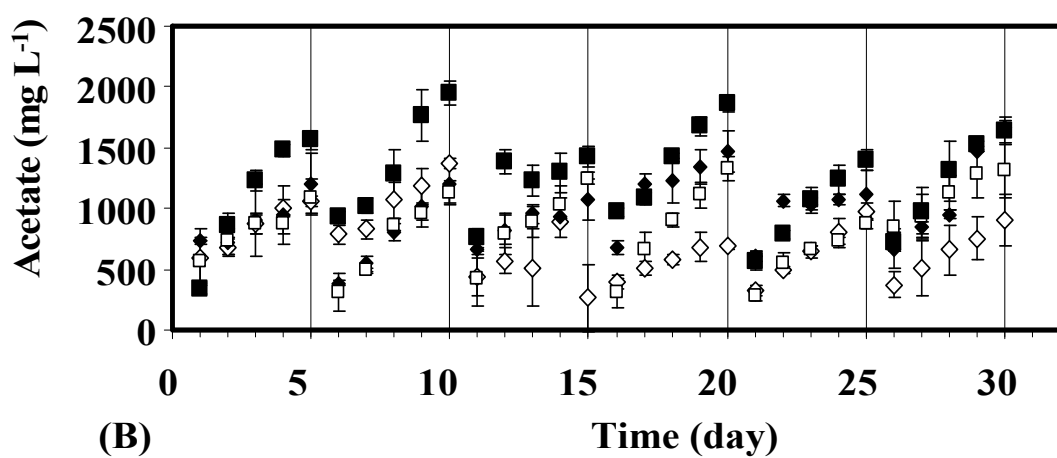
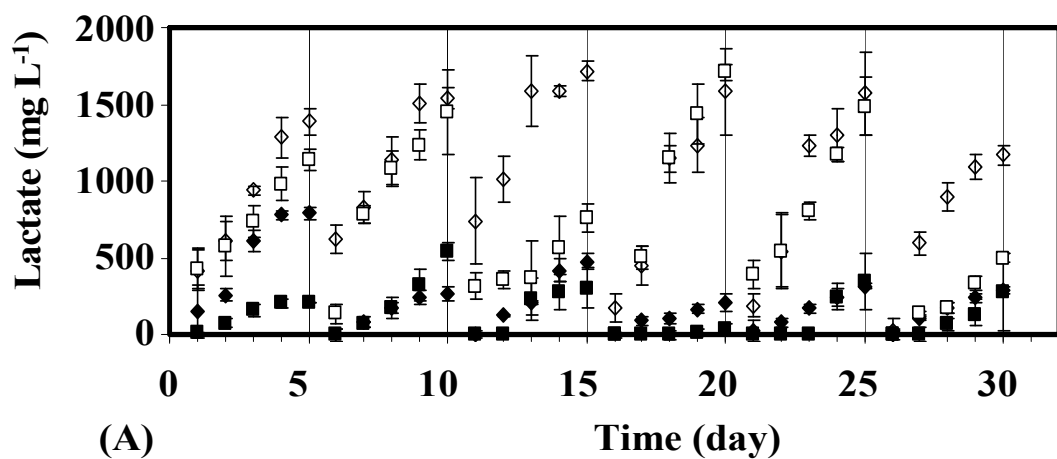
granular and flocculated cultures are from the same origin and received the same treatment with LA, granules disintegration decreased the initial glucose degradation rate.

Post hoc analyses (Tukey's multiple means comparison test at 95% confidence interval) indicated that the initial glucose degradation rates were similar for LA-treated samples and their corresponding controls.

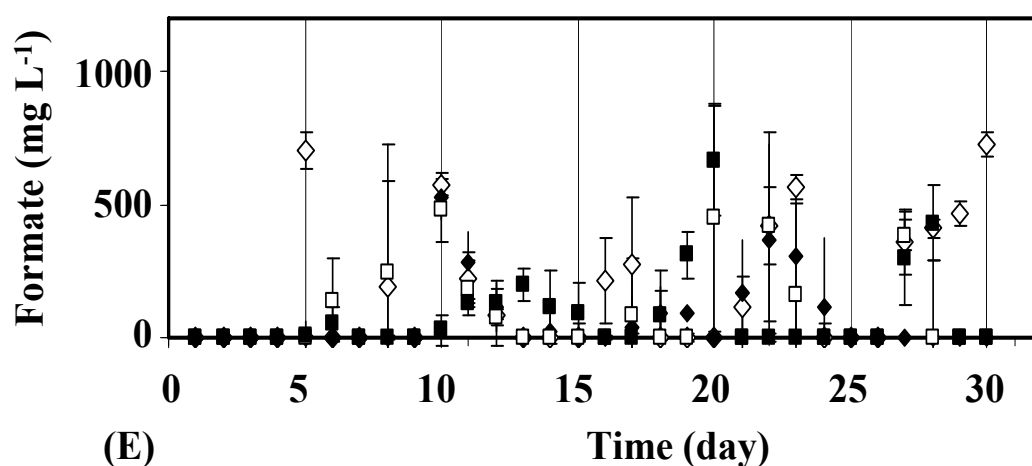
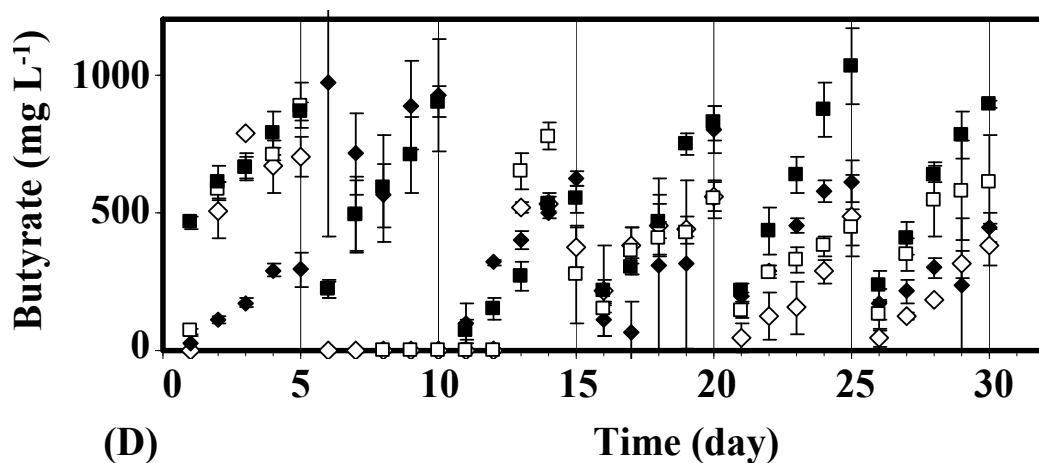
### **8.3.6 Volatile fatty acids production**

The major VFAs detected were acetate, butyrate, propionate, lactate, and small quantities of formate; VFAs were produced in control and LA-treated samples of granular and flocculated cultures (Figure 8.6). Both granular and flocculated cultures produced various quantities of lactate (Figure 8.6A). LA-incubated cultures produced less lactate than their corresponding control samples. Flocculated cultures generally produced less lactate than the granular cultures. The maximum lactate concentrations in control cultures were  $1720 \pm 60 \text{ mg L}^{-1}$  (G3; Day 15) and  $1710 \pm 50 \text{ mg L}^{-1}$  (S3; Day 20) in granular and suspended culture, respectively. The maximum lactate concentrations in LA-treated cultures were  $780 \pm 41 \text{ mg L}^{-1}$  (G1; Day 5) and  $540 \pm 60 \text{ mg L}^{-1}$  (S1; Day 10) in granular and flocculated culture, respectively.

All cultures (granular or flocculated cultures) produced acetate under control and experimental conditions (Figure 8.6B), but increasing acetate concentrations were measured in all LA-treated samples of the granular and flocculated cultures in comparison to control samples. Flocculated cultures produced more acetate than granular culture in both



—◇— Granular control      —◆— Granular + LA  
 —□— Flocculated control      —■— Flocculated + LA



—◇— Granular control      ◆ Granular + LA  
 - □ - Flocculated control      ■ Flocculated + LA

Figure 8.6 VFAs production profiles at varying LA incubation periods in cultures receiving  $2,000 \text{ mg L}^{-1}$  LA plus  $5,000 \text{ mg L}^{-1}$  glucose. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

control and LA-treated samples. The maximum acetate concentrations were  $1370 \pm 40 \text{ mg L}^{-1}$  on day 10 and  $1320 \pm 220 \text{ mg L}^{-1}$  on day 30 in the control samples of the granular and suspended culture, respectively. The maximum acetate concentrations were  $1900 \pm 100 \text{ mg L}^{-1}$  on day 20 and  $1950 \pm 100 \text{ mg L}^{-1}$  on day 10 in the LA-treated samples of the granular and flocculated culture, respectively.

All cultures produced butyrate under the controlled and experimental conditions (Figure 8.6C). The flocculated cultures which were incubated with LA for 0, 20 and 25 days produced more butyrate than the granular cultures. The maximum butyrate concentrations under the control conditions were  $790 \pm 2 \text{ mg L}^{-1}$  on day 2 and  $780 \pm 50 \text{ mg L}^{-1}$  on day 14 in the control samples of the granular and flocculated cultures, respectively. The maximum butyrate concentrations under the experimental conditions were  $980 \pm 550 \text{ mg L}^{-1}$  on day 6 and  $1030 \pm 140 \text{ mg L}^{-1}$  on day 25 in the LA-treated granular and flocculated cultures, respectively. Flocculated cultures incubated with LA for 0, 5, and 15 days did not produce butyrate and less than  $250 \text{ mg L}^{-1}$  was produced on day 23 and 24.

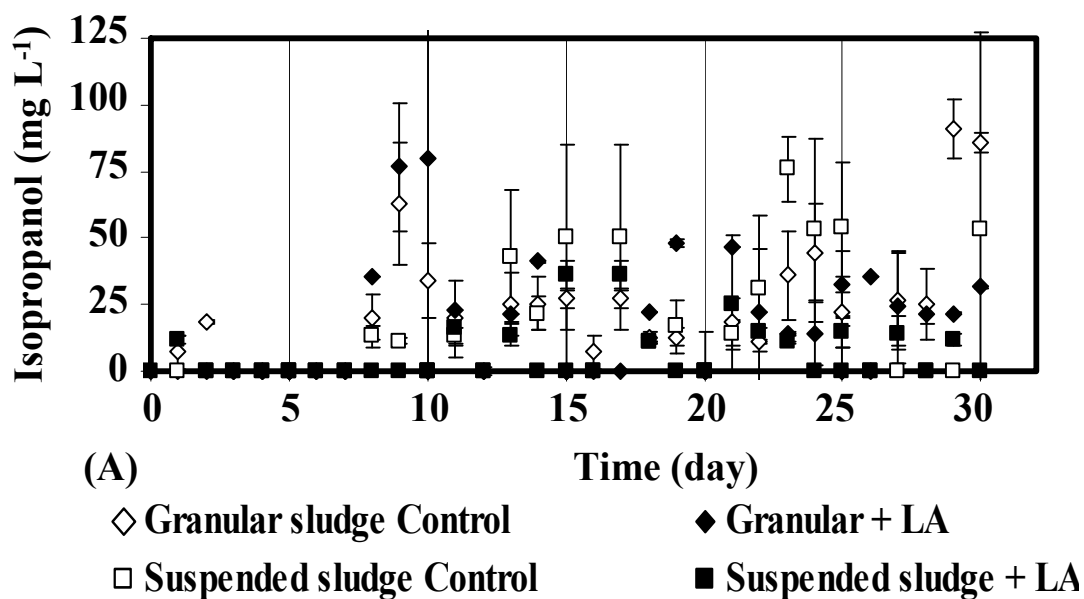
Propionate was also produced in all cultures under control and experimental conditions. For both granular and flocculated cultures, propionate production decreased in samples incubated with LA for 20 and 25 days in comparison to the same samples incubated for 0 to 15 days (Figure 8.6D). In fact, LA-incubated flocculated cultures did not produce propionate after incubated for 0, 5 and 15 days. No other specific trends were observed for propionate production. The maximum propionate concentrations were  $640 \pm 90 \text{ mg L}^{-1}$  on day 10 and  $580 \pm 20 \text{ mg L}^{-1}$  on day 15 in the control samples of the granular and suspended culture, respectively. The maximum propionate concentrations were  $520 \pm 225 \text{ mg L}^{-1}$  on day 15 and  $505 \pm 28 \text{ mg L}^{-1}$  on day 15 in the LA-treated granular and flocculated cultures, respectively.

Formate was produced in small quantities in cultures incubated for more than 5 days. Most of the formate produced was detected in the control samples of the granular culture, however, no specific trend was observed in glucose control or LA-treated cultures.

### 8.3.7 Alcohols production

The alcohols detected were isopropanol and propanol (Figure 8.7). The concentrations of isopropanol produced in all cultures were less than 100 mg L<sup>-1</sup>. Cultures incubated for 0 and 5 days produced less than 25 mg L<sup>-1</sup> of isopropanol. Generally, control cultures produced more isopropanol than LA-treated cultures.

Propanol was produced in control and LA-incubated granular and flocculated cultures. A maximum propanol concentration 290±8 mg L<sup>-1</sup> was detected in the LA-treated granular culture incubated for 5 days and the average concentration was approximately 200 mg L<sup>-1</sup> in LA-treated granular cultures. The average propanol concentration was approximately 100 mg L<sup>-1</sup> in control flocculated and granular cultures and in the LA-treated flocculated culture.



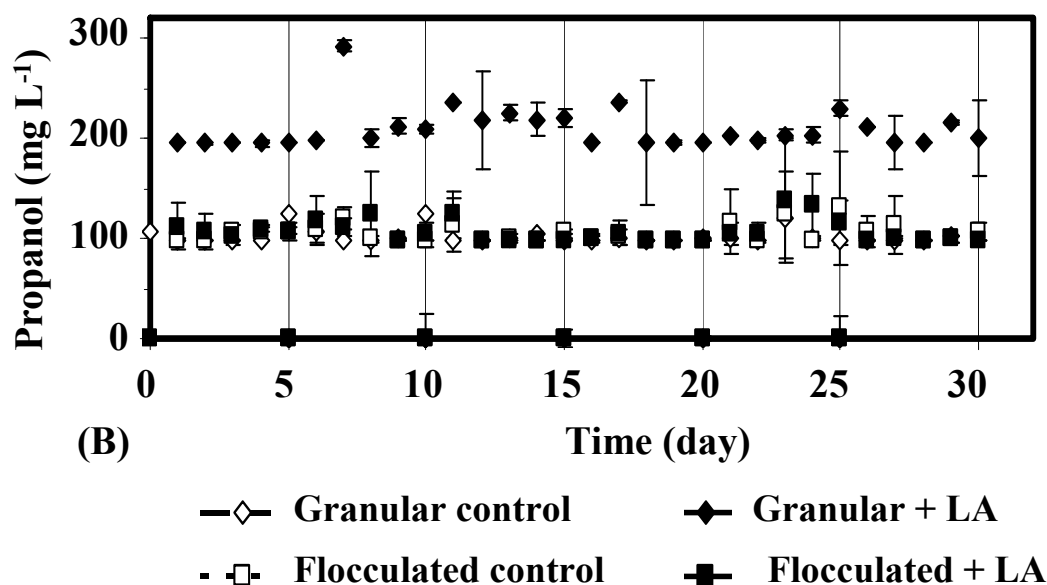


Figure 8.7 Alcohols (A) isopropanol and (B) Propanol production profiles at different LA incubation periods in cultures receiving 2,000 mg L<sup>-1</sup> LA plus 5,000 mg L<sup>-1</sup> glucose. (Values shown are mean  $\pm$  standard deviation for triplicate samples).

### 8.3.8 Flux balance analysis (FBA) of H<sub>2</sub> production

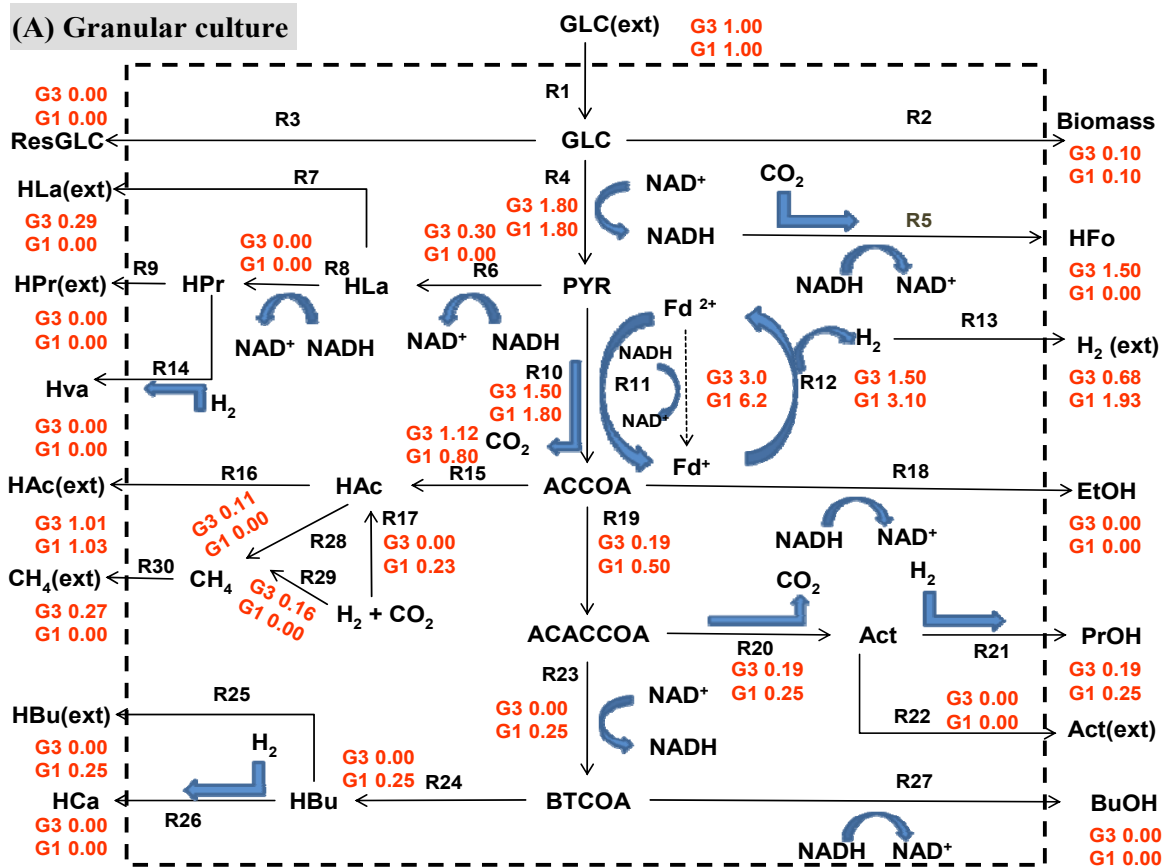
Mass balance analyses of the metabolic fluxes during maximum H<sub>2</sub> yield were performed according to the metabolic reactions network of the model shown in Figure 3.3. H<sub>2</sub> production data from glucose-controls (G3 and S3) and LA-treated (G1 and S1) granular and flocculated cultures were analyzed (MetaFluxNet software, Version.1.8.6.2). Stoichiometries of the reactions (R1 to R30) used in the model are shown in Table 3.9.

The input included the external substrate (glucose) and the measured by-products (gases, VFAs, and alcohols). Figure 8.8 show the output of the FBA of the by-products data on the day when the maximum H<sub>2</sub> yields in the control and LA-treated cultures were observed.

Careful examination of Table 8.4 reveals that the order of H<sub>2</sub> consumption (mol H<sub>2</sub> per mol glucose) for the different conditions examined is as follows: G1 “granular + glucose + LA” (1.17) > S3 “suspended glucose control” (1.07) > G3 “granular control” (0.83) >



S1 “suspended + glucose + LA” (0.18). It is also clear that in the granular cultures, homoacetogenic  $H_2$  consumption became active only after methanogens were inhibited with LA. The glucose-control granular cultures (G3) consumed 0.83 mol of  $H_2$  [ $4 \times 0.16 = 0.64$  mol in  $CH_4$  production plus 0.19 mol in propanol production (R21)] compared to 1.17 mol of  $H_2$  in LA-treated samples [ $4 \times 0.23 = 0.92$  mol in the homoacetogenic reaction (R17) and 0.25 mol in propanol production (R21)]. In the glucose-control suspended culture (S3),  $H_2$  consumption was 1.06 mol, and proceeded via the homoacetogenic reaction (R17) which consumed ( $4 \times 0.22 = 0.88$  mol) plus 0.18 mol in propanol production (R21). Notice that homoacetogenic  $H_2$  consumption in LA-treated suspended culture was zero.



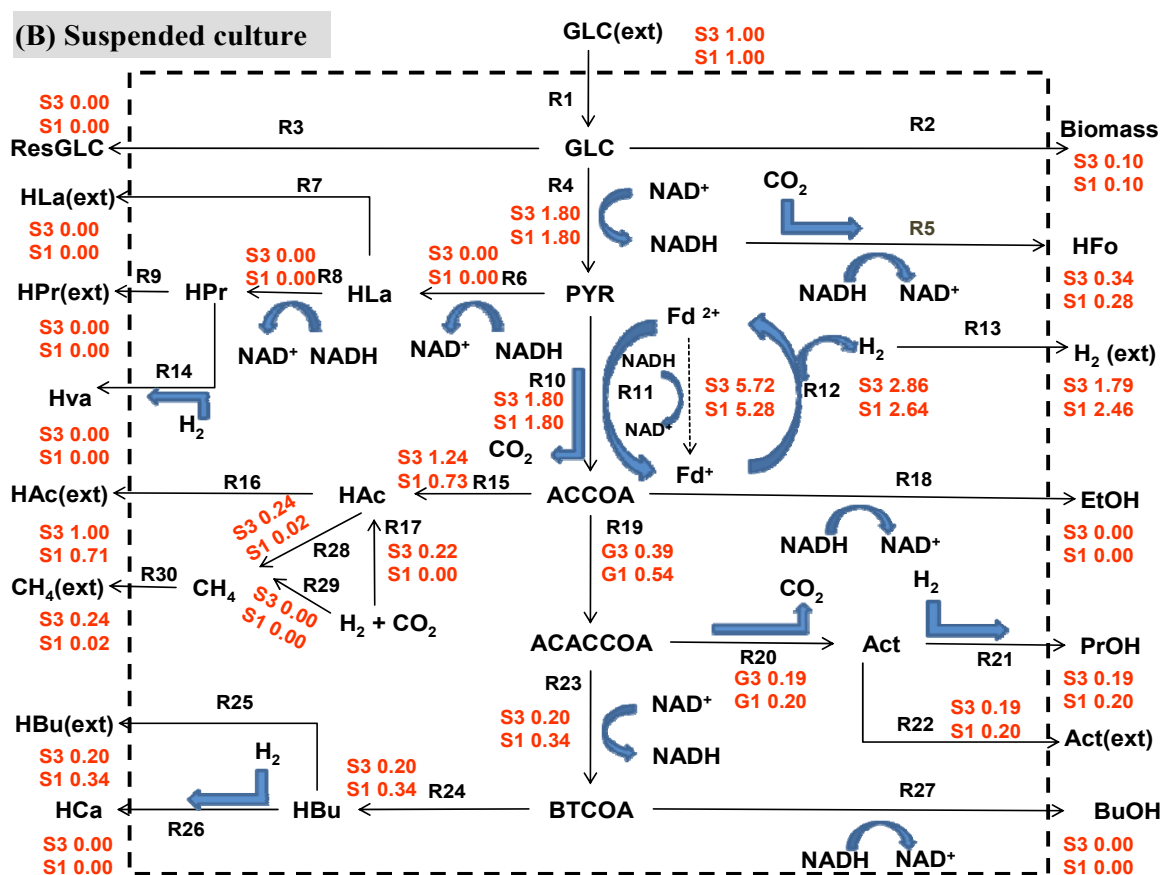
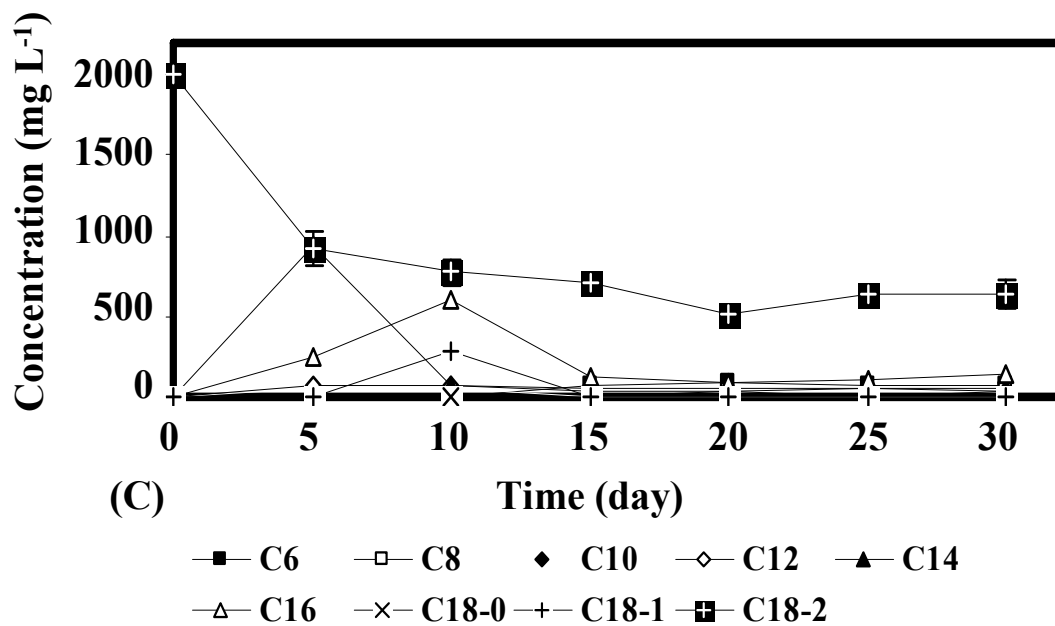
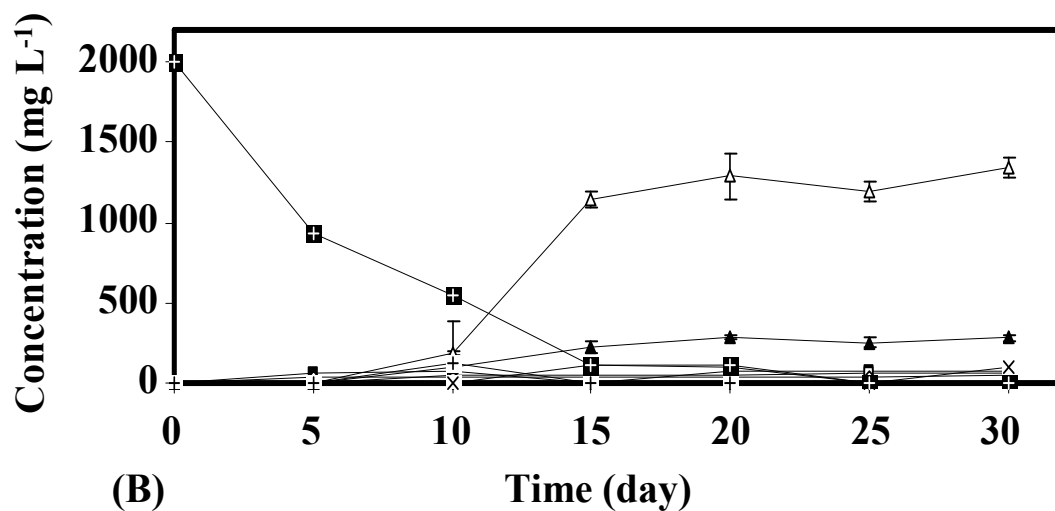
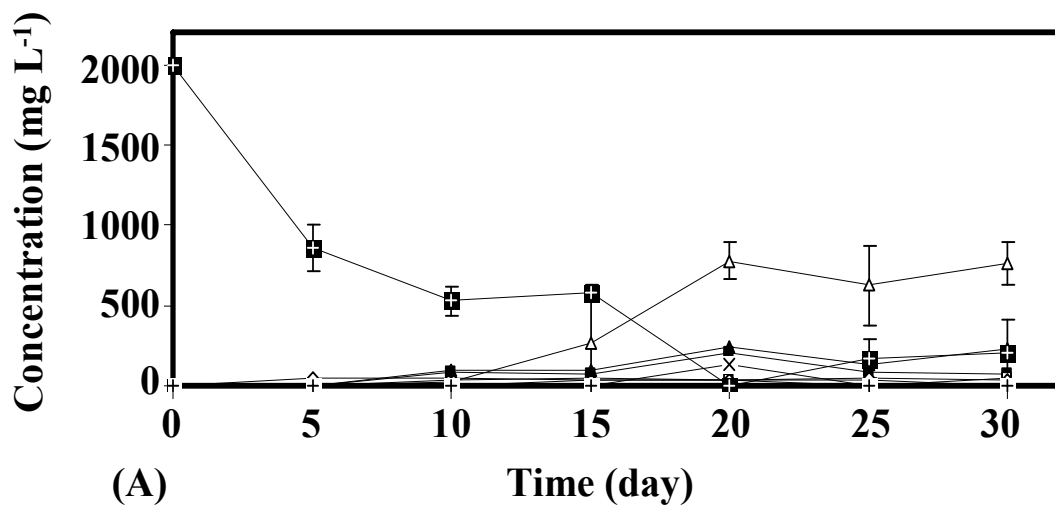


Figure 8.8 Flux balance analysis for the glucose control and glucose plus LA treated samples of (A) granular and (B) flocculated cultures using by-products data set for the maximum H<sub>2</sub> yield.

### 8.3.9 Linoleic acid (LA) degradation

Linoleic acid degradation and the products of  $\beta$ -oxidation reactions were monitored every 5 days. The profiles of LA (2,000 mg L<sup>-1</sup>) degradation and the formation of shorter LCFA by-products are shown in Figure 8.9. LA was degraded in the LA-control samples of granular (G2) and flocculated (S2) cultures to undetectable levels within 20 and 25 days, respectively (Figure 8.9B and D). The LA degradation rates in the LA-control samples were 29 $\pm$ 5 (G2) and 21 $\pm$ 7 (S2)  $\mu$ g LA  $\cdot$  mg VSS<sup>-1</sup>  $\cdot$  day<sup>-1</sup> (Table 8.3).



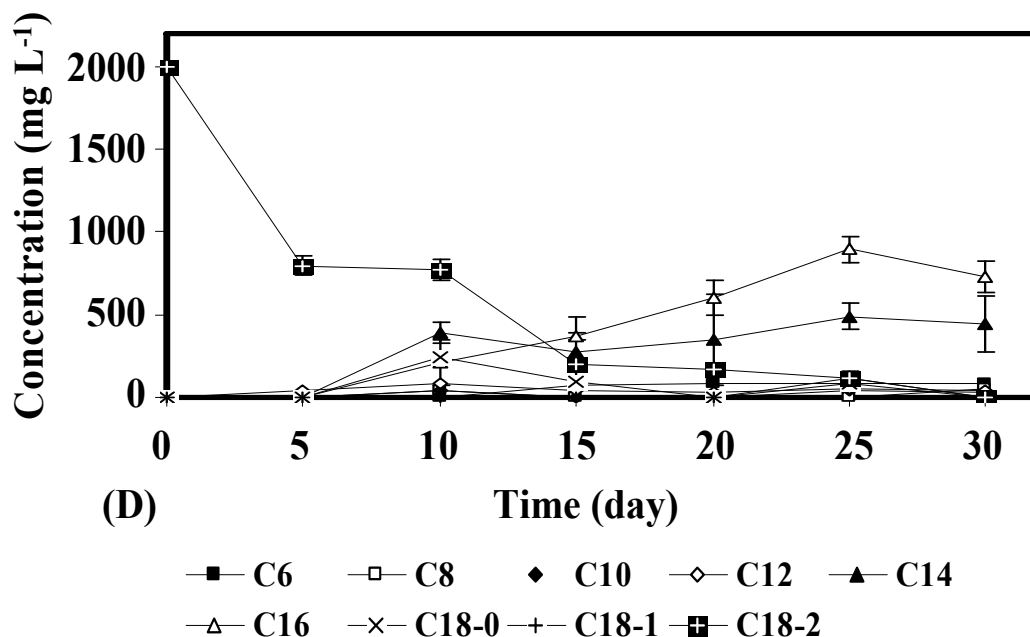


Figure 8.9 Long chain fatty acids profiles at varying times in LA (2,000 mg L<sup>-1</sup>) control samples and glucose plus LA (2,000 mg L<sup>-1</sup>) samples of granular and flocculated cultures. (A) G1= granular + glucose + LA, (B) G2 = granular LA control, (C) S1 = flocculated + glucose + LA, and (D) S2 = flocculated LA control. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

Table 8.3 LA degradation rate (Values shown are mean  $\pm$  standard deviations for triplicate samples).

LA degradation rate ( $\mu\text{g LA} \cdot \text{mgVSS}^{-1} \cdot \text{day}^{-1}$ )			
Granular culture		Suspended culture	
LA Control (G2)	Glucose + LA (G1)	LA Control (S2)	Glucose + LA (S1)
28.97 $\pm$ 4.81 <sup>a</sup>	15.45 $\pm$ 6.70 <sup>b</sup>	21.08 $\pm$ 6.92 <sup>b</sup>	5.88 $\pm$ 1.11 <sup>c</sup>

Note: <sup>a</sup> and <sup>b</sup> indicate significantly different means.

PA, MA and LUA were detected with maximum levels observed at different times. The maximum levels were detected for the following LCFAs: PA (S2: Day 25 and G2: Day 25), MA (S2: Day 25 and G2: Day 25) and LUA (S2: Day 10 and G2: Day 20). Maximum levels of 900 $\pm$ 80 mg L<sup>-1</sup> PA , 490 $\pm$ 80 mg L<sup>-1</sup> MA , and 83 $\pm$ 37 LUA mg L<sup>-1</sup> were observed in the LA-control flocculated cultures (S2), and 1340 $\pm$ 63 mg L<sup>-1</sup> PA,

290±13 mg L<sup>-1</sup> MA, and 49±15 LUA mg L<sup>-1</sup> were detected in LA-control granular cultures (G2). The PA concentration reached a stable value by Day 15 in LA-control granular culture (G2), but not until Day 25 in the LA-control flocculated culture (S2).

LA degradation was also observed in granular and flocculated cultures fed glucose and treated with LA (G1 and S1) (Figure 8.9A and C). By-products of the  $\beta$ -oxidation process consisted of acetate plus PA, MA and LUA (Figure 8.9). LA concentrations detected on day 30 were 640±85 and 210±200 mg L<sup>-1</sup> in flocculated (S1) and granular (G1) culture, respectively. In comparison, the LA degradation rate in LA-controls of the flocculated (S2) and granular (G2) culture were 5.9±1.1 and 15.5±6.7  $\mu\text{g LA} \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$ , respectively (Table 8.3).

Samples of glucose-fed and LA-treated granular culture (G1) accumulated more PA than the corresponding samples of the flocculated culture (S1). Maximum levels of 780±120 mg L<sup>-1</sup> and 600±40mg L<sup>-1</sup> of PA were observed on day 20 and day 25 in granular (G1) and flocculated (S1) cultures, respectively. Trace and transitory concentrations of stearic acid (SA, C18:0) were observed (130±75 mg L<sup>-1</sup>) on day 20 in the granular culture (G1). Similarly, trace and transitory concentrations of oleic acid (OA, C18:1) were observed (280±320 mg L<sup>-1</sup>) on day 10 in the flocculated cultures (S1).

The maximum MA concentrations observed were 240±55 mg L<sup>-1</sup> on day 20 and 940±430 mg L<sup>-1</sup> on day 5 in the granular (G1) and flocculated (S1) cultures, respectively. Low concentrations of LUA were detected throughout the incubation period. Peak levels were 73±9 and 48 ±18 mg L<sup>-1</sup> on day 5 and day 25 in flocculated (S1) and granular (G1) cultures, respectively.

Generally, the flocculated culture maintained higher concentrations of LA in comparison with the granular culture (Figure 8.10A and B). Differences in the H<sub>2</sub> yield and its trend in the flocculated and granular cultures were parallel to the increase in the

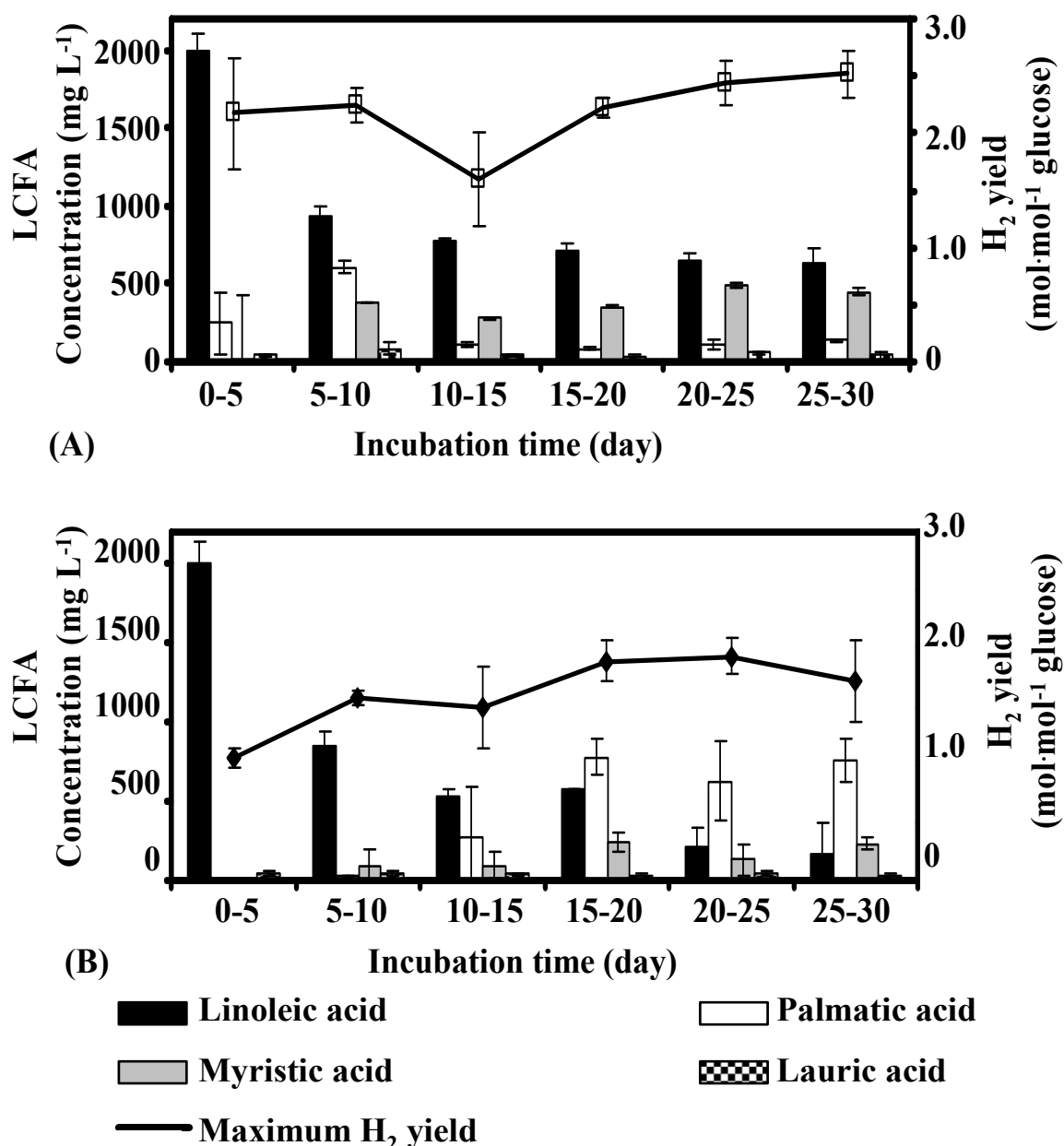


Figure 8.10 Hydrogen yields as a function of LA  $\beta$ -oxidation by-products in (A) suspended and (B) granular cultures incubated with LA. (LCFA levels are the maximum concentrations during the analysis. (Values shown are mean and standard deviation for triplicate samples).

difference between the concentrations of LA in the two cultures particularly during the incubation period (25-30 days). The decrease in  $H_2$  yield in the granular culture from  $1.9 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose during the incubation periods (15-20 and 20-25) to  $1.6 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose was associated with a decrease in LA concentrations ( $< 500 \text{ mg L}^{-1}$ ). Note the  $H_2$  consumption in the granular culture despite the increase in the PA concentrations during the final incubation period.

### **8.3.10 Principal Components Analysis (PCA)**

Principal Components Analysis (PCA) was applied in order to visualize the main differences and similarities in data related to LA degradation and its by-products in flocculated (S1 [LA + glucose] and S2 [LA-control]) and granular (G1 [LA + glucose] and G2 [LA-control]) cultures from the same source. The data set consisted of nine variables (LA degradation by-products: C6 to C18:2) and 36 cases (conditions). All variables have the same scale. The first two principal components (PC1 and PC2) contained 59% of the total variability present in the data set. PC1 was mostly correlated with LA, PA, MA, and hexanoic acid (HA) while PC2 was correlated with CAA, LUA, and OA. The plane PC1 versus PC2 (Figure 8.11) shows that the flocculated cultures (S1 and S2) and the granular culture (G1 and G2) behaved differently. For flocculated culture (S), the samples from the first two incubation periods (e.g. 5S1 and 10S1) contributed to much of the variability because their scores were high on both PC1 and PC2 (-3.1 and -3.25 on PC1 and 3 and 1.5 on PC2, respectively). In the case of granular culture (G), the samples incubated for 20 or 25 days contributed much of the variability because their scores were especially high on PC1 (2.5) and their scores on PC2 were also large

compared to other incubation periods. The loading of a variable is related to its variability.

Notice that for any of the four lines (S1, S2, G1, and G2) depicted in Figure 8.11, both flocculated and granular cultures were in the same direction of positive scores on PC1. However, the observations for flocculated and granular cultures were in opposite directions on PC2 as the incubation period increased. Only the granular culture (G1 and G2) was dislocated in the direction of positive scores on PC2 whereas flocculated culture (S1 and S2) was dislocated in the direction of negative scores.

Analysis of the score map (Figure 8.11) in connection with its corresponding loading plot (Figure 8.12) can identify influential variables. Projections of the variables (individual LCFA) on the principal components (Figure 8.12) indicate that LA, PA, MA,

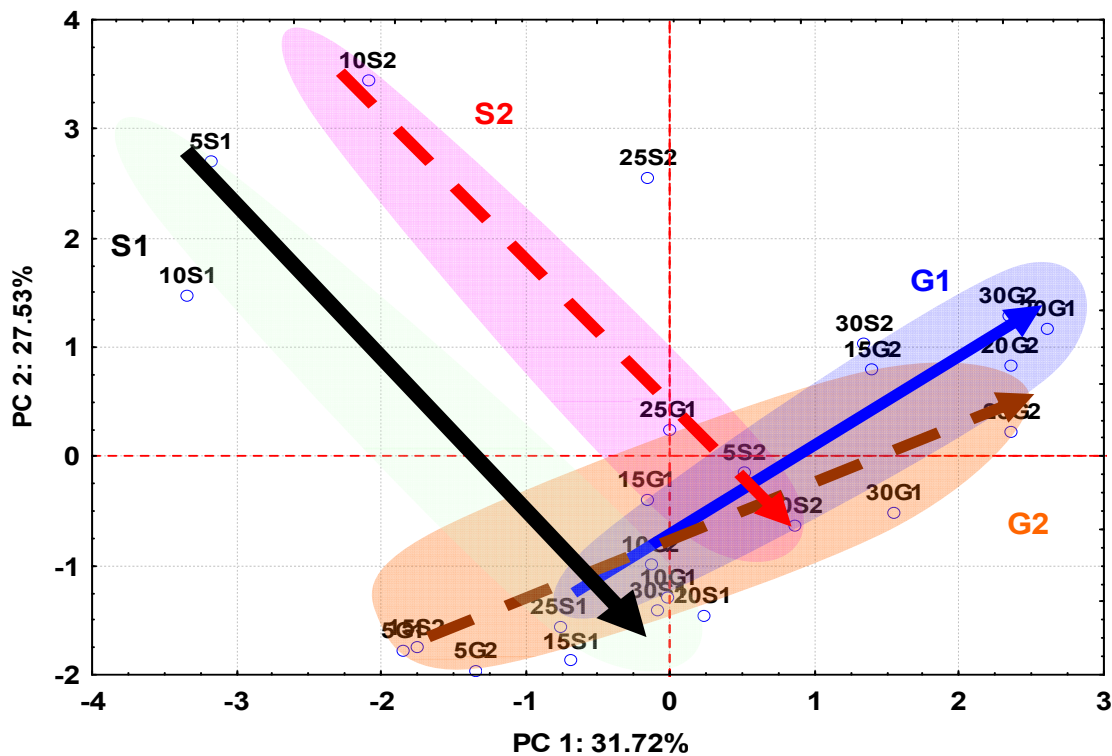


Figure 8.11 Principal components analysis of LCFAs extraction data set for granular (G) and flocculated (S) culture. (S1 and G1= glucose plus LA treated cultures; S2 and G2 = LA-control cultures).



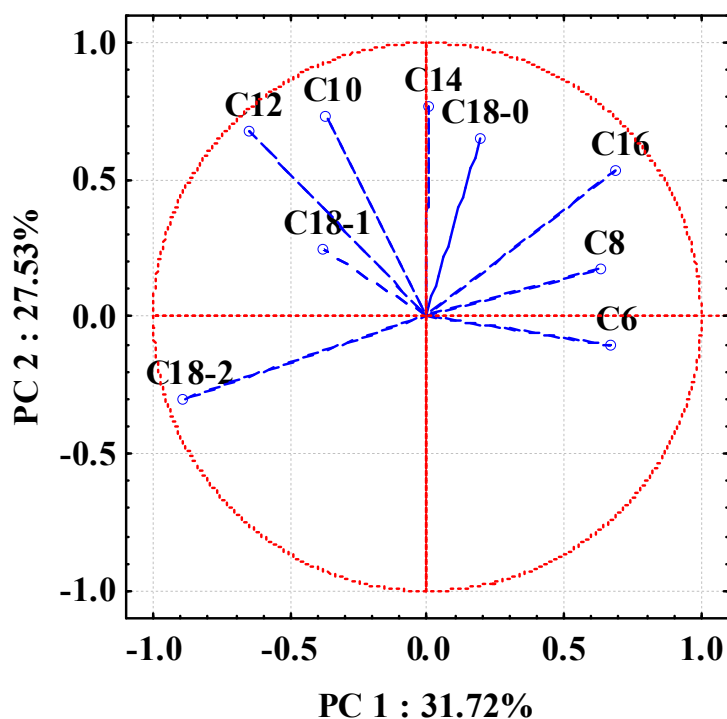


Figure 8.12 PCA loading map for LA degradation by-products data set.

LUA, and CAA are the most important by-products that contributed to the variation explained by the two principal components

PCA was also applied to the data set of fermentation by-products ( $H_2$ ,  $CH_4$ , VFAs and alcohols). The data set consisted of 10 variables (fermentation by-products:  $H_2$ ,  $CH_4$ , lactate, acetate, propionate, butyrate, formate, isopropanol and propanol) and four cases (conditions: glucose-fed and LA-treated (S1 and G1) and glucose control (S3 and G3) samples of the flocculated and granular cultures. The first two principal components contained 62% (PC1) and 27% (PC2), accounting for 89% of the total variability present in the data set. PC1 was correlated with  $H_2$ , acetate, butyrate, and propanol whereas PC2 was correlated with  $CH_4$ , propionate, and isopropanol. The plane PC1 versus PC2 (Figure 8.13) shows that samples of granular and flocculated cultures fed glucose and treated with LA (S1 and G1) were closer to each other than to their corresponding glucose

controls (S3 and G3). The glucose control cultures (S3 and G3) were very far from each other; this separation indicate different behaviors of granular and flocculated cultures under the inhibition of LA and low pH.

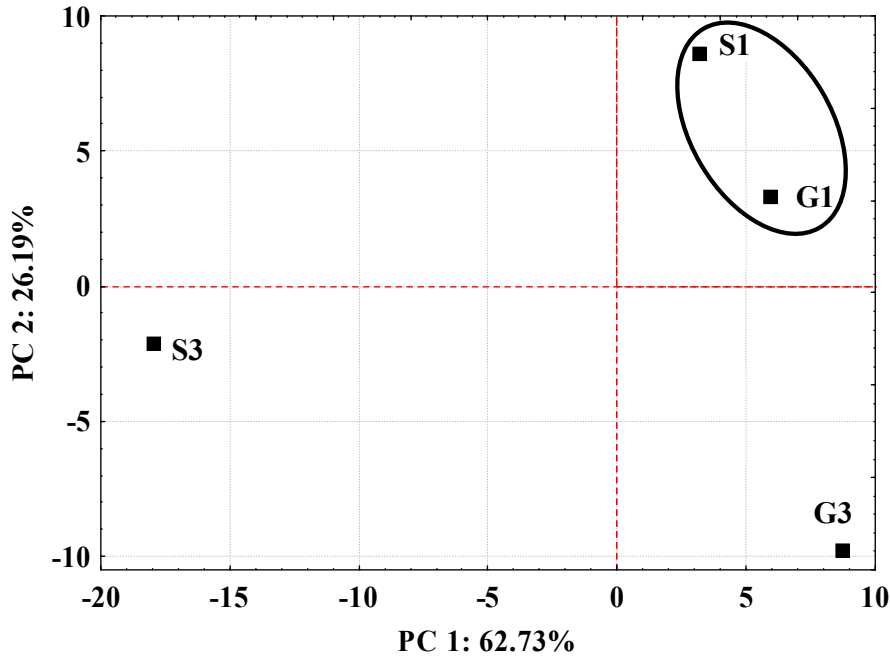
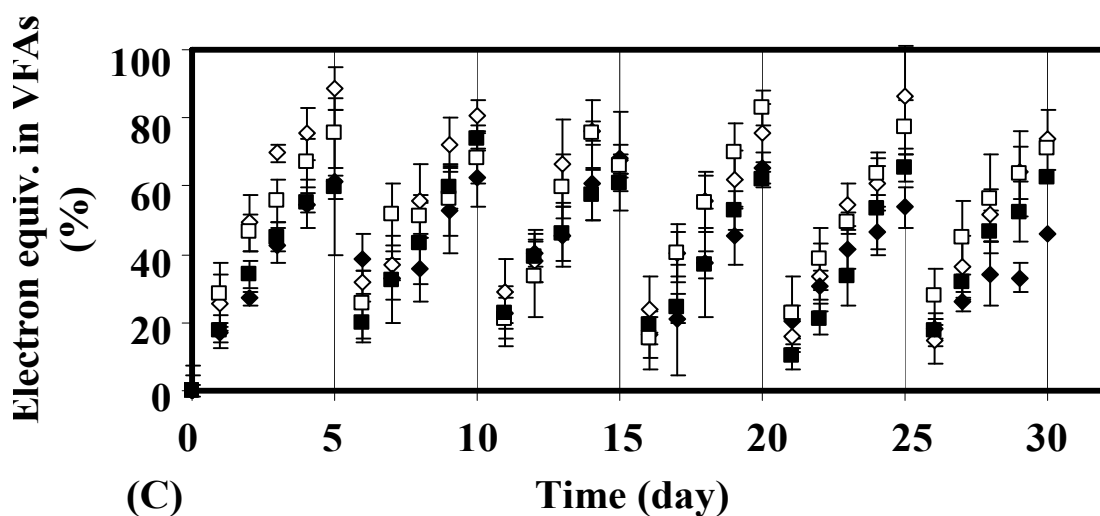
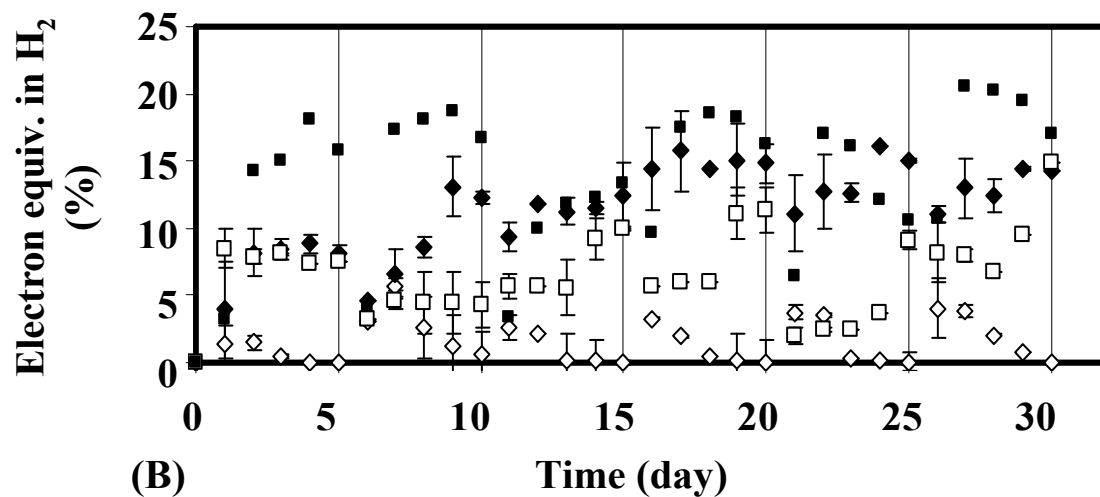
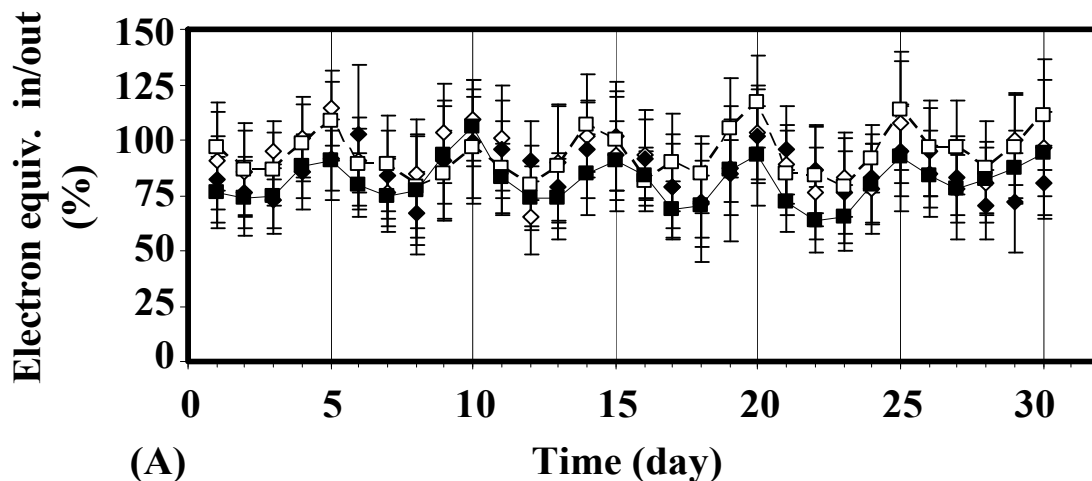


Figure 8.13 Principal component analysis of fermentation by-products data set for control and LA treated samples of granular and flocculated culture. (S1 and G1= glucose plus LA-treated flocculated and granular culture, respectively. S3 and G3 = glucose or LA-control samples of flocculated and granular culture, respectively).

### 8.3.11 Electron mass balance

The distribution of the electrons in glucose to the various by-products was assessed by an electron mass balance. The acetate electrons derived from LA degradation in the LA-control samples (S2 and G2) were subtracted from the total number of acetate electrons measured in the glucose plus LA-treated samples (S1 and G1). Figure 8.14 shows the electron balance for the glucose control (G3 and S3) and glucose plus LA-treated (G1 and S1) granular and flocculated cultures for incubation periods of 5 to 25 days. Flocculated



—◇— Granular control      —◆— Granular + LA  
 - -□- Flocculated control      - -■- Flocculated + LA

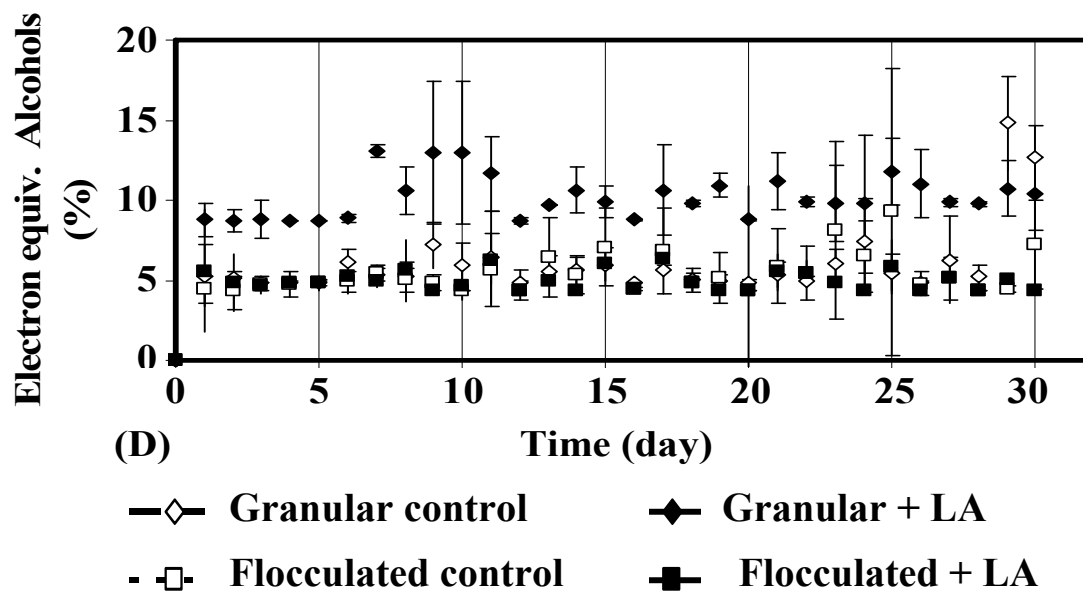


Figure 8.14 Electron mass balance for glucose controls (S3 and G3) and glucose plus LA-treated ( $2,000 \text{ mg L}^{-1}$ ) (S1 and G1) granular and flocculated culture incubated for 0, 5, 10, 15, 20, 25, and 30 days. (Values shown are mean  $\pm$  standard deviations for triplicate samples).

culture fed glucose and treated with LA (S1) synthesized more than 20% of the electron equivalents available in glucose into  $\text{H}_2$ . Most of the electron equivalents ended up tied to VFAs (Figure 8.14C). The LA-treated granular culture (G1) channeled a higher percentage of electron equivalents towards production of alcohols compared to the flocculated culture (S1).

### 8.3.12 Microbiological analysis

The molecular diversity of the bacterial community was investigated by nested-PCR of the 16S rRNA gene using the T-RFLP method. Samples of the untreated original culture, the glucose control (S3 and G3) and glucose plus LA-treated samples (S1 and G1), and LA-control (S2 and G2) of the flocculated and granular cultures were analyzed. *Hae* III and *Hha* I T-RFLP profiles were obtained and the raw data sets are comprised of peaks

reflecting the size in base pairs (bp) of terminal restriction fragments (T-RFs) and the area of each peak measured in fluorescence units. The relative abundance of each band detected was determined by calculating the area of each peak as a percentage of the total area. The T-RFLP gel images are shown in Figure 8.15. The number of relatively abundant bacterial species, estimated by the mean richness determined from 16S rRNA T-RFLP *Hae* III profiles, are plotted as percentage (relative abundance) against fragment size in (bp) in Figure 8.16 and 8.17 for flocculated and granular cultures, respectively. The original (untreated) culture (lane a in Figure 8.15A and Figure 8.16A) was dominated by: *Clostridium* and *Acetobacter* (band 16, bp 227) 29%; *Corynebacterium* sp. (band 3, bp 63) 13%; *Bacillus* and *Bacteroides* sp. (band 2, bp 62) 12%; *Clostridium*, *Bacteroides*, *Eubacterium*, and *Geobacterim* sp. (band 14, bp 217) 8%; *Clostridium*, *Flavobacterium*, and *Lactobacillus* sp. (band 19, bp 254) 7%; *Bacillus* and *Clostridium* sp. (band 15, bp 222) 6%; and *Clostridium*, *Eubacterium*, and *Enterococcus* sp. (band 24, bp 301) 5%.

*Clostridium*, *Bacillus*, and *Brevibacillus* sp. (band 5, bp 223) dominated the glucose control samples of the suspended culture (lane b in Figure 8.15A and Figure 8.16D) by 49%, followed by *Clostridium* sp. (band 4, bp 220) 35%, and *Bacteroides* sp. (bands 1 and 2, bp 56 and 58) 11%. In the flocculated cultures which were fed glucose plus LA (lane c in Figure 8.15A and Figure 8.16B), the percentage of the *Bacteroides* sp. (band 2, bp 60) increased to 52%, the percentage of the *Clostridium* sp. (band 5, bp 223) decreased to 25%, *Bacillis*, *Brevibacillus*, and *Clostridium* sp. (band 4, bp 220) accounted for 11%, and *Moorella theromacetcum* (band 1, bp 56) was 5%.

The LA control for flocculated culture [S2] is shown in lane d in Figure 8.15A and Figure 8.16C, This culture showed greater diversity in comparison to the glucose control

[S3] and glucose plus LA [S1] cultures. *Clostridium* and *Bacillus* sp. (band 19, bp 221) decreased to 19%, *Clostridium* and *Bacteroides* sp. (band 17, bp 202) was 11%, *Acinetobacterim* sp (band, bp 251) was 9%, *Bacillus*, *Brevibacillus*, and *Eubacterium* (band 20, bp 233) was 7%, *Alicyclobacillus* (band 9, bp 78) was 8%, *Moorella theromacetcum* (band 4, bp 57) was 4%, and *Corynebacterium* sp. (band 5, bp 63) was 7%. *Brevibacillus* sp. (band 13, bp 222) dominated the glucose control of the granular culture [G3] (lane e in Figure 8.15A and Figure 8.17D) at 37%, followed by *Capnocytophaga* sp. (band 1, bp 41) 27%, *Clostridium* sp. (bands 14, bp 302) was 7%, *Corynebacterium* sp. (band 4, bp 69) 5.08%, and *Bacteroides* sp. (band 2, bp 61) accounted for 5%.

The granular culture fed glucose and LA [G1] (lane f in Figure 8.15A and Figure 8.17B) included *Bacillus* sp. (band 3, bp 61) 37%, *Cytophaga* and *Eubacterium* sp. Was (band 11, bp 222) 19 %, *Clostridium*, *Butyrivibrio*, *Eubacterium*, *Fusobacterium*, *Gloeobacter*, and *Lactobacillus* sp. (band 15, bp 276) accounted for 6%, *Brevibacillus* sp. (band 10, bp 217) 4 %, *Bacteroides* and *Flavobacterium* sp. (band 13, bp 261) 2%, and *Bacteroides* (band 14, bp 266) was 2%.

The LA control of granular culture [G2] (lane g in Figure 8.15A and Figure 8.17C) also showed greater diversity in comparison to the corresponding cultures, glucose control [G3] and glucose plus LA [G1]. *Bifidobacterium* sp. (band 21, bp 257) 36%, *Actinobacillus*, *Bacteroides*, and *Flavobacterium* sp. (band 22, bp 261) 27%, *Acinetobacterim*, *Bacillus*, and *Cytophaga* sp (band 20, bp 251) 5 %, *Flavobacterium* sp. (band 8, bp 179) 5%, *Propionibacterium* sp. (band 3, bp 55) 4%, *Bacteroides* (band 11,

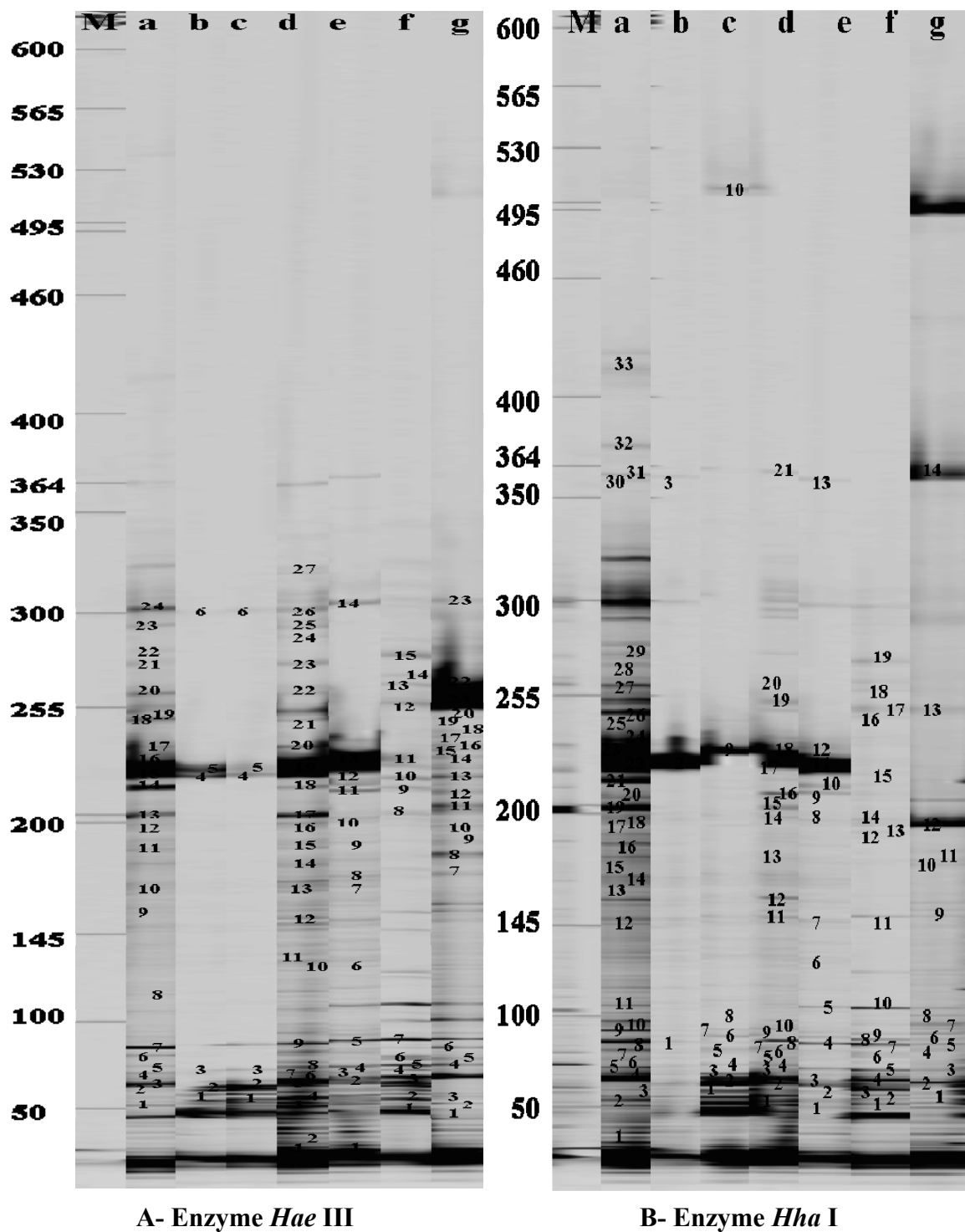


Figure 8.15 Bacterial community profiles in suspended and granular cultures (Lanes a = original untreated culture, b and e = glucose control of flocculated and granular cultures (S3 and G3), respectively, c and f = flocculated and granular cultures fed glucose plus LA (S1 and G1), respectively, d and g = LA control of flocculated and granular cultures (S2 and G2), respectively). Numbers on the lanes indicate the bands identified.

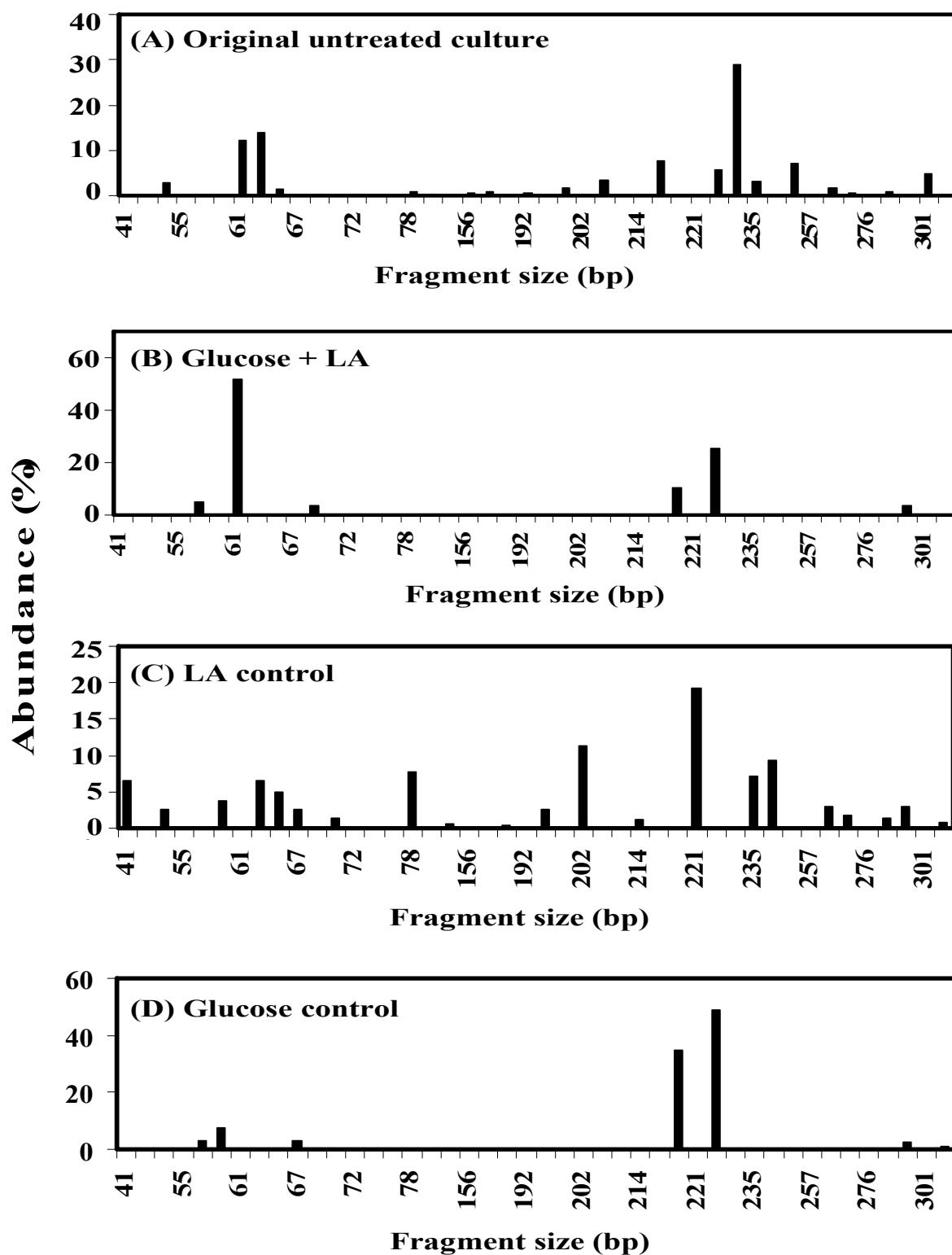


Figure 8.16 Comparison of original culture and flocculated culture based on the fragment size detected with the enzyme *Hae* III. (A) original untreated culture, (B) glucose-fed and LA-treated, (C) LA control, and (D) glucose control cultures).



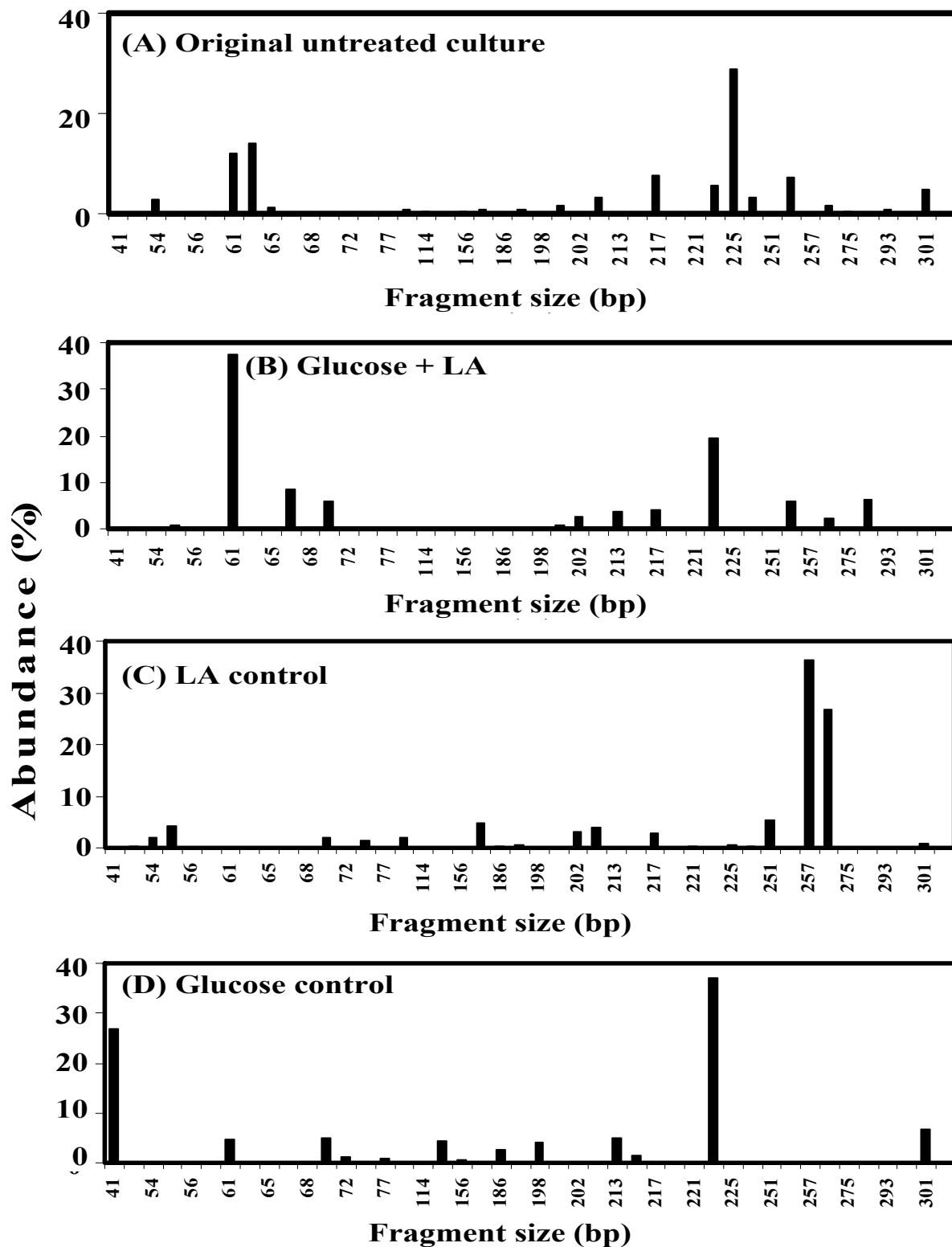
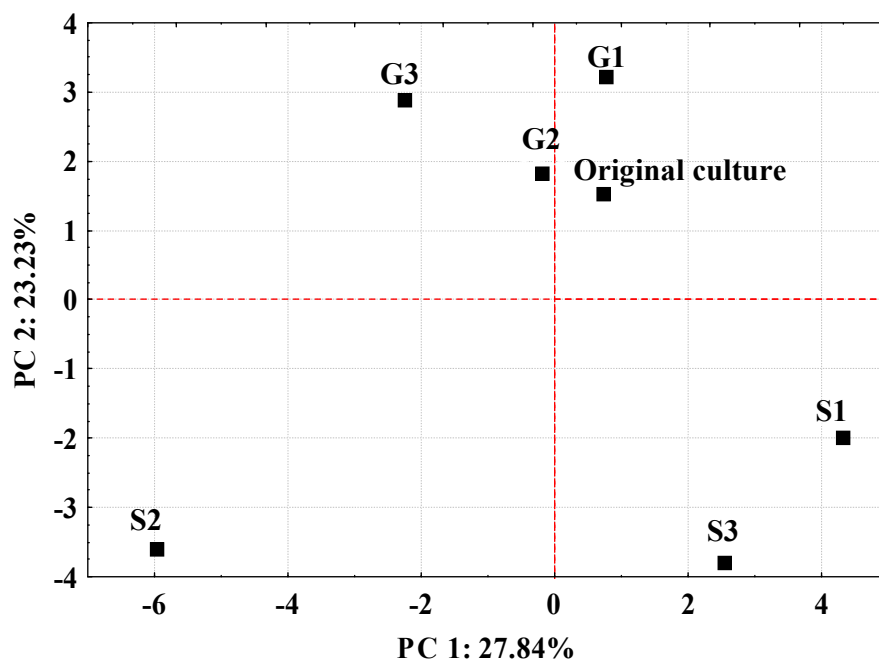


Figure 8.17 Comparison of original culture and samples of granular culture based on the fragment size detected with the enzyme *Hae* III. (A) original untreated culture, (B) glucose-fed and LA-treated, (C) LA control, and (D) glucose control cultures).

bp 202) 3%, *Clostridium* and *Eubacteria* sp. (band 13, bp 217) 3%, and *Bacteroides* (band 2, bp 54) 2%.

Multivariate statistical analysis (PCA) was applied to the T-RFLP's profiles using the enzyme *Hae* III data set. Three PCs accounted for 69% of the variance in this data set. The score maps of the PCA detected similarity between the original untreated culture and all samples of the granular culture (G1, G2, and G3) as shown in Figure 8.18A. The LA-control of the granular culture (G2) was closer to the original culture than were the glucose control (G3) and glucose plus LA-fed samples (G1) on the plane of PC1-PC2. On the score map of the planes of PC2-PC3 and PC1-PC3, the LA-treated cultures of the granular culture (G1 and G2) were closer to each other than to the glucose control sample (G3).



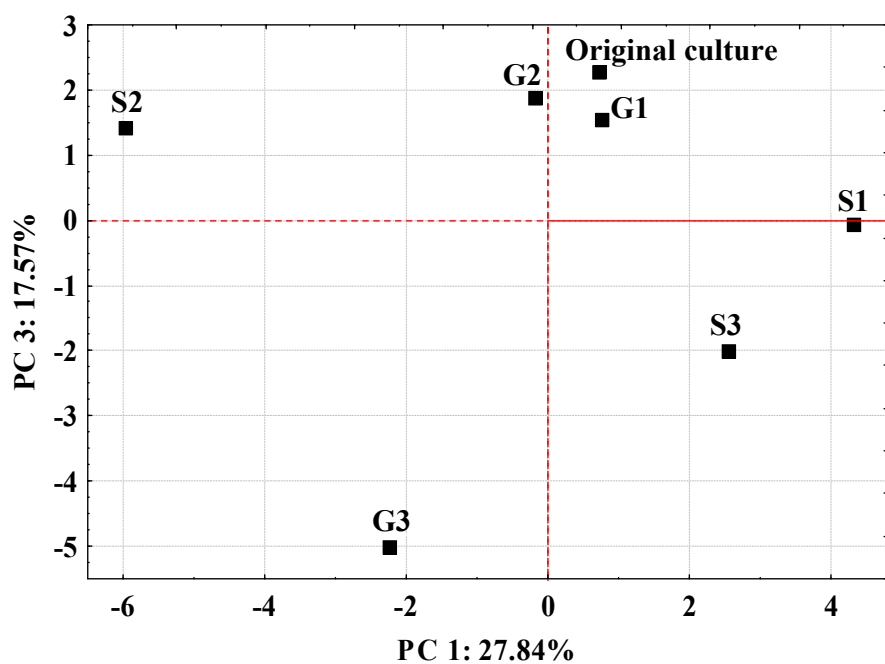
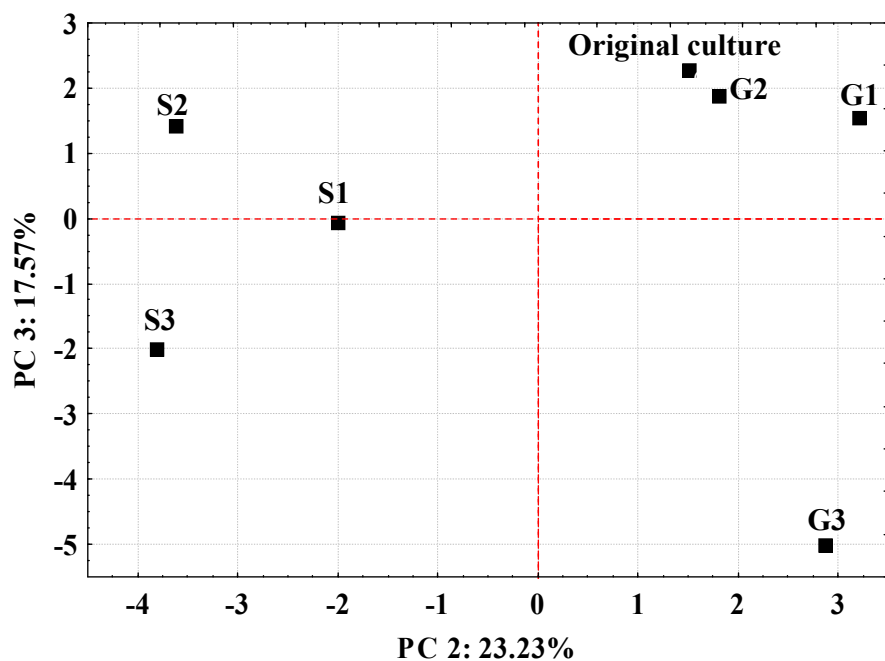


Figure 8.18 Score maps of the PCA of the bacterial community profiles from the T-RFLP analysis data set for flocculated (S) and granular (G) cultures (original culture = untreated culture, S3 and G3 = glucose control of flocculated and granular cultures, S1 and G1 = flocculated and granular cultures fed glucose plus LA, S2 and G2 = LA control of flocculated and granular cultures).

## 8.4 DISCUSSION

Mixed anaerobic culture has the potential to produce  $H_2$  and to provide a cheap and easy-to-maintain alternative to pure culture. In comparison with other pre-treatment methods, LCFAs enhanced the yield of  $H_2$  from anaerobic mixed culture communities to more than  $3 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ glucose}$  as reported in chapter 4, 5, and 6. The physical structure of the microbial culture can be either flocculated or granulated. Anaerobic culture grown in suspension would perform differently from granules because of the difference in the microbial environment (Jia and Fang, 1999). To date, no research has compared  $H_2$  production from flocculated and granular cultures under the stress due to the presence of LCFAs. Moreover, no studies investigated long-term  $H_2$  production under this stressful condition (exposure to LCFAs) and addressed the question of probable adaptation of methanogens to LCFAs. In this work, controlling  $H_2$  consumption by the combined effect of LA-treatment ( $2000 \text{ mg L}^{-1}$ ) with an initial pH of 5, and diverting electron equivalents towards  $H_2$  was demonstrated over various incubation periods in both granular and flocculated cultures derived from the same source. In addition, this study profiled the microbial communities in each condition using T-RFLP analysis. The  $H_2$  and methane production profiles suggest that methanogens showed more inhibition in flocculated structure than in the granulated structure. These observations are in agreement with previous results reported by Hwu *et al.* (1996). The maximum  $H_2$  yield observed in suspended culture ( $2.5 \pm 0.2 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ glucose}$ ) was higher than that observed in granular culture ( $1.9 \pm 0.2 \text{ mol } H_2 \cdot \text{mol}^{-1} \text{ glucose}$ ).

Post hoc analyses (multiple mean comparisons using Tukey's test at 95% confidence interval) indicated that the maximum yield of  $H_2$  in LA-treated samples was significantly

different from that in control samples for both granular and flocculated cultures (Table 8.1). The maximum yield of  $H_2$  in LA-treated samples of the flocculated culture was also significantly different from LA-treated samples of granular culture (Table 8.1). The significant difference in  $H_2$  yield confirms a difference in the flow of electrons in the granular and flocculated cultures. The local micro-environments in the granules and flocs differ, and can affect  $H_2$  metabolism differently because in granular culture, for example, the microorganisms respond to the local LCFA concentration at the interface between liquid and granule, not to the bulk concentration (Rinzema *et al.*, 1994).

Methanogenic activity was higher in the granular culture compared to flocculated culture. Thus,  $H_2$  consumption by hydrogenotrophic methanogens was minimized in flocculated cultures compared to the granular culture. These results are consistent with findings reported previously by Hwu *et al.* (1996). These differences can be explained by the fast interspecies  $H_2$  transfer between syntrophs and methanogens in the dense granules (de Bok *et al.*, 2004; Stams, 1994; Batstone *et al.*, 2006) compared to slower transfer in the dispersed flocs of microorganisms in flocculated cultures. Granules form densely compacted aggregates of a layered structure with each layer occupied by a different trophic group. Acetoclastic methanogens occupy the internal core of the granule, whereas acetogenic and hydrogenotrophic microorganisms occupy the second (intermediate) layer. The outer layer includes acidogenic, sulfate-reducing and hydrogenotrophic bacteria (Fang *et al.*, 1994; Guiot *et al.*, 1992; MacLeod *et al.*, 1990; Quarmby and Forster, 1995). Methanogenic microorganisms in the core of granules are shielded from LCFAs, so are more protected from their toxic effects in comparison with methanogenic microorganisms in the flocs of the flocculated culture. Methanogens are

protected within a granular structure from chemical attack as well as heat treatment (Danko *et al.*, 2008). These researchers reported that flocculated cultures are more adversely affected than granular cultures during heat treatment.

The flux of compounds transferred between microorganisms depends on the surface area of the producing bacterium, the concentration gradient, and the distance between the organisms (de Bok *et al.*, 2004). Thiele *et al.* (1988) reported an increase in surface area upon disrupting large microbial structures. According to Batstone *et al.* (2006), the  $H_2$  flux is inversely proportional to the distance between the syntrophs and methanogens. Schmidt and Ahring (1995) reported that the average distance between cells in intact granules was approximately 2 to 3  $\mu m$  compared to 10  $\mu m$  after disintegration. LCFAs adsorb to the cell wall and form a coating layer, which hinders the transport of substrates from the bulk liquid to the cells. However, the diffusion of  $H_2$  through the LCFA layer is faster than other substrates, thus it is immediately converted into methane (Pereira *et al.*, 2003). The combined effects of granule disintegration, LA, low pH, and LA degradation by-products reduced the  $H_2$  consumption and increased the  $H_2$  yield. Note that increasing the surface area upon the disintegration of granules means that there is greater contact with LCFAs, and hence, stronger inhibition of the cell. Hydrogen consumption by other  $H_2$ -consuming organisms, such as homoacetogenic bacteria, was observed with longer incubation times, but not in the LA-treated flocculated culture. This finding is in agreement with previously reported studies that syntrophs are a major sink for  $H_2$  (Batstone *et al.*, 2006) even under inhibited methanogenesis (Nollet *et al.*, 1997; Wejima *et al.*, 2002). Hence, dense biomass granules favor this mechanism of  $H_2$  consumption (Dinamarca and Bakke, 2008). The results of the FBA confirmed this finding since the

order of homoacetogenic H<sub>2</sub> consumption (in mol of H<sub>2</sub> per mol of glucose) was G1 (0.92) > S3 (0.88) > G3 (0.0) = S1 (0.0). The homoacetogenic H<sub>2</sub> consumption in the granular culture was activated only after LA inhibited the methanogens, whereas in the flocculated culture it occurred only in the control samples (likely due to the inhibitory effect of the increased VFAs production). Hydrogen consumption by solventogenesis ranged between 0.19 and 0.25 mol of H<sub>2</sub> per mol of glucose in both flocculated and granular cultures.

Although [FeFe]-hydrogenase is the most efficient H<sub>2</sub>-producing enzyme it can also consume H<sub>2</sub>, depending upon the particular cellular circuitry (Hallenbeck, 2009). Therefore, many organisms which contain “uptake” hydrogenases show low H<sub>2</sub> yield. Richard *et al.* (1999) reported two membrane-bound hydrogenases, which are involved in periplasmic H<sub>2</sub> uptake. For example, four polypeptides form integral membrane electron transfer components (Sawers, 2005) and regulate part of the hydrogenase enzyme in *E. coli* (Self *et al.*, 2004; Sawers, 2005). Therefore, preventing the correct assembly of the uptake hydrogenases in the cytoplasmic membrane could decrease H<sub>2</sub> oxidation and account for the observed increases in H<sub>2</sub> production. Mathews and Wang (2009) reported a 4-fold increase in H<sub>2</sub> yields after suppressing the activity of H<sub>2</sub> uptake hydrogenases. Moreover, genetic knockout of a subunit in an uptake hydrogenase in *E. aerogenes* was able to increase the H<sub>2</sub> yield from 0.65 to 0.78 mol H<sub>2</sub> / mol glucose (Zhao *et al.*, 2009).

The mechanism of LCFAs inhibition is attributed to physicochemical interactions between the LCFA and the cell wall (Galbraith *et al.*, 1971, 1973; Rinzema *et al.*, 1994). Microbial cells within the flocs of flocculated cultures would be in greater contact with LCFAs than the cells in granular structure. In granular structures, abiotic (extracellular

polymers and inert) materials constitute up to 60% of the VSS (Rezania *et al.*, 2006) and would adsorb a large portion of the applied LCFA; therefore, a cell in the granular structure would have less contact with LCFAs. A similar phenomenon was observed in rumen bacteria where fat preferentially adheres to fibrous plant particles rather than bacterial cells (Devendra and Lewis, 1974; Galbraith *et al.*, 1971). This alleviates the cell surface interactions with LCFA and allows normal nutrient uptake by the microbes (Devendra and Lewis, 1974).

In addition to interspecies H<sub>2</sub> transfer in granules, direct extracellular electron transfer from cell-to-cell through a c-type cytochrome was recently reported by Summers *et al.* (2010). These researchers reported that electrically conductive microbial aggregates containing species of *Geobacter* are able to transfer electrons externally through electrically conductive pili. The microbiological analysis using T-RFLP revealed that 8% of the original culture was *Geobacter metallireducens*, a mesophilic Gram-negative strict anaerobe, which grows chemoorganotrophically and can consume acetate, propionate, butyrate, and ethanol (Garrrity *et al.*, 2005).

Higher H<sub>2</sub> yields were observed in LA-treated culture in comparison to the controls in both granular and flocculated cultures. After 15 days of incubation, the conversion efficiency of glucose to H<sub>2</sub> increased with the incubation period and was 55%, 61%, and 63 % (based on the maximum biologically possible level of 4.0 mol H<sub>2</sub>·mol<sup>-1</sup> glucose) in flocculated culture incubated with LA for 15, 20, and 25 days, respectively. Nevertheless, no particular corresponding trend in H<sub>2</sub> yield was observed with the increasing incubation times.



The results suggest that for the incubation period examined (0 to 25 days), the methanogenic inhibition due to LA was not reversed in granular or flocculated culture. However, a slight increase in methane production with longer incubation periods was observed in granular and flocculated cultures incubated for 5 to 25 days. This is likely due to the growth of the surviving hydrogenotrophic methanogens. Methane production in control samples was significantly different from LA-treated samples for both flocculated and granular culture (Table 8.2). Methane production in flocculated cultures was significantly different from the production in control samples of granular culture. Methane production in LA-treated flocculated culture was significantly different from LA-treated granular culture (Table 8.2).

Acidogens were inhibited more in the flocculated culture compared to granular culture. This is indicated by the higher loss of initial acidogenic activity measured by glucose initial degradation rate because LCFAs interfere with cellular functions responsible for glucose degradation (Lewis, 1992). Accumulation of the fatty acids acetate, butyrate, propionate and lactate indicate the inhibition of acetogenic bacteria due to the inhibition of acetoclastic and, to a lesser extent, hydrogenotrophic methanogens. Notice that LA-treated samples accumulated more VFAs in comparison to their corresponding controls.

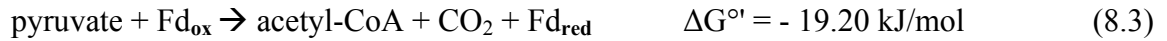
Proton-reducing acetogenic bacteria that degrade LCFAs live in syntrophy with methanogens because they consume acetate and H<sub>2</sub> and cause the  $\beta$ -oxidation reactions to become thermodynamically favourable. The presence of *Bacillus*, *Clostridium*, *Eubacterium*, *propionibacterium*, and *Bacteroides* in the LA-control samples of granular and flocculated cultures is in agreement with the findings of Kemp *et al.* (1975), Verhulst *et al.* (1985), Fujimoto *et al.* (1993), and Hatamoto *et al.* (2007).

Whether or not methanogens would adapt to the inhibitory effect of LCFAs is still subject to investigation. The permanent toxic and antibacterial effects of LCFAs (Hanaki *et al.*, 1981; Rinzema *et al.*, 1994; Angelidaki and Ahring, 1992) were well accepted until recently; however, several reports have indicated that inhibition by LCFAs is reversible and not permanent (Alves *et al.*, 2001; Pereira *et al.*, 2004, 2005). They reported acclimation of anaerobic culture to LCFAs, which enhanced the degradation of OA. The results of this study agree with reports of the irreversibility of LCFAs inhibition. The inhibitory effect of LCFAs increases with the degree of instauration (number of the carbon-carbon double bonds in the carbon chain) (Rinzema, 1988). Note that, for example, Shin *et al.* (2003) pointed out the high degradation rate of OA in comparison to that of SA and PA.

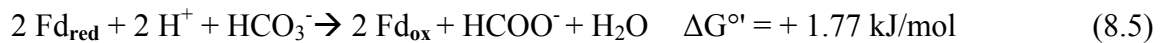
Syntrophic fatty acid-oxidizing bacteria are affected severely by the inhibition of hydrogenotrophic methanogens (Schink, 1997). Compare the rates of LA degradation in LA-control and glucose plus LA-treated samples; its degradation was slower in glucose-treated culture because of much greater inhibition of syntrophic bacteria due to the high hydrogen partial pressure ( $P_{H_2}$ ) and the accumulation of acidogenic by-products. Furthermore, VFAs produced during the acidogenic reactions impose a synergistic inhibitory effect with LCFAs. Post hoc analyses (Tukey's statistical significance comparison test at 95% confidence interval) indicate that the LA degradation rate in LA-control samples was significantly different from the samples fed glucose plus LA for both granular and suspended cultures (Table 8.3).

The partial pressure of hydrogen ( $P_{H_2}$ ) inside the microbial flocs, where most activity takes place, may be very different than the  $P_{H_2}$  in the bulk gas phase (Thiele and Zeikus, 1988). This might explain the continued  $H_2$  production even under high  $P_{H_2}$ .

The observed build-up of formate (between 100 to 600 mg L<sup>-1</sup> in the controls and LA treated samples through the entire fermentation) indicates prevalence of unusual conditions due to decoupling of the fermentative and methanogenic microorganisms. The following discussion is related to the findings of Grobick and Stuckey (1989). Formate could be produced in two main pathways; either from the degradation of pyruvate by pyruvate formate lyase (Thauer *et al.*, 1977) or indirectly from the degradation of pyruvate by ferredoxin oxidoreductase:

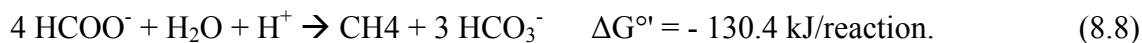


The ferredoxin is then be regenerated by producing  $H_2$  (Eq. 8.4) or formate (8.5):



These reactions are catalyzed by hydrogenase and  $\text{CO}_2$  reductase, respectively. Kinetically, formate (Eq. 8.5) may be major electrons sink for fermentative bacteria in mixed cultures, particularly, species which do not contain hydrogenase (Grobick and Stuckey, 1989). Formate could be degraded to  $H_2$  and  $\text{CO}_2$  (Eq. 8.6) or transformed to methane (Eq. 8.7 and 8.8) (Zeikus, 1977; Marty, 1984), the thermodynamics of the reactions involved:





Methanogenesis from formate (Eq. 8.8) is thermodynamically favourable and independent of hydrogen partial pressure ( $P_{\text{H}_2}$ ). It is more energetic than either methanogenesis from  $\text{H}_2/\text{CO}_2$  (Eq. 8.7) or cleavage of formate to  $\text{H}_2/\text{CO}_2$  (Eq. 8.6). The kinetic of methanogenesis from formate is faster than from acetate, and even from  $\text{H}_2$  (Schauer *et al.*, 1982; Daniels *et al.*, 1984).

Insight into the behaviour of the microbial community under the various conditions examined might explain the experimental results. The greater diversity of fermentative and acidogenic bacteria (i.e. *Bacteroides* 52%, *Clostridium* 25%, and *Bacillus*, *Brevibacillus*, and *Clostridium* 11%, and *Moorella thermoacetica* 5%) seen in the flocculated culture fed glucose and LA (S1) could account for the higher  $\text{H}_2$  yield. Notice that *Bacteroides* can produce  $\text{H}_2$  because they contain  $\text{H}_2$ -evolving hydrogenase similar to *Clostridium* sp. (Madigan *et al.*, 2000). Moreover, *Moorella thermoacetica* (originally isolated as *Clostridium thermoacetica*) is a spore-forming acetogen, which can grow autotrophically on  $\text{H}_2/\text{CO}_2$ . However, the FBA did not reveal any homoacetogenic  $\text{H}_2$  consumption in the flocculated culture fed glucose and incubated with LA (S1). Conversely, in granular culture fed glucose plus LA (G1), the dominant species were *Bacillus thermoleovorans* 37%, *Eubacterium* 19 %, *Clostridium*, *Butyrivibrio*, *Fusobacterium*, and *Gloeobacter* sp. 6%, and *Bacteroides* and *Falvobacterium* 4%. Notice that *Eubacterium* is a  $\text{H}_2$ -oxidizing,  $\text{CO}_2$ -reducing, Gram-positive, non-spore-forming anaerobic bacterium (Tanner *et al.*, 1981), and that MFA revealed homoacetogenic  $\text{H}_2$  consumption in the granular culture (G1). This difference in the

composition of the microbial communities could be one of many, and might explain the difference in H<sub>2</sub> yield between the flocculated (S1) and granular (G1) cultures.

In the granular cultures, the percentage of *Bacteroides* decreased from 36% in the LA control samples (G2) to 5% in the glucose control samples (G3), and to 2% in the granular culture fed glucose plus LA (G1). Concurrently, *Bacillus* increased from 0.0% in G2 (LA-control) to 37% in G1 (the culture fed both glucose and LA). Moreover, *Eubacterium* increased from 0.0% in G3 (glucose control) to 3% in G2 (LA control) and to 19 % in G1 (the culture fed both glucose plus LA). The percentage of *Clostridium* sp. was almost the same (6.0%) in all samples (G1, G2, and G3) of the granular culture. Nieman (1954) reported that the inhibition of *Bacillus subtilis* was transient and restricted to the early period of incubation.

The species dominated in the LA control flocculated cultures were different from those dominated in the granular cultures (Table 8.4). Although, for example, *Bifidobacteria* are Gram-positive strict anaerobes which ferment sugars to acetic and lactic acids with no gas (Ward and Bora, 2009) but it dominated in LA control of the granular culture. Moreover,

Table 8.4 Dominant species in LA control samples of suspended and granular cultures.

Suspended culture (S2)			Granular culture (G2)		
<u>Species</u>	<u>Abundance (%)</u>	<u>Gram-type</u>	<u>Species</u>	<u>Abundance (%)</u>	<u>Gram-type</u>
<i>Anaeroplasma</i>	11.93	-	<i>Bifidobacterium</i>	36.23	+
<i>Acinetobacter</i>	9.35	-	<i>Actinobacillus s</i>	26.78	-
<i>Alicyclobacillus</i>	7.64	+	<i>Flavobacterim</i>		-
<i>Corynebacterium</i>	6.62	-			

*Alicyclobacillus* are gram-positive, spore forming bacteria which grow at temperatures between 25 and 65°C and pH of 2.5 to 6.0. It would be surprising if all the

microorganisms in Table 8.4 did not play a role in LCFAs degradation because of their high relative abundance in the LA control cultures; however, to date none of these microorganisms have been reported to be involved in LCFA degradation.

PCA was used to detect similarities and differences in the profiles of LA degradation by-products in flocculated and granulated culture from the same origin. The PCA confirmed clearly that there were differences between the profiles of PA, MA, LUA, CAA, CAPA and HA in flocculated culture in comparison to granular culture, and highlighted the impact of the incubation period. In Figure 8.11, notice the differences in the clustering and the direction of the lines (S1 and G1: glucose and LA treated) and (S2 and G2: LA controls) cultures. The directions of these lines indicate the effects of the incubation periods. In the flocculated culture (S1 and S2), short incubation periods contributed much of the variation explained by PC1 and PC2; notice the high scores for the incubation periods of 5 and 10 days, (i.e., 5S1 and 10S1) on PC1 and PC2. In contrast to flocculated culture, the longest incubation periods examined (20 and 25 days) contributed much of the variability explained by PC1 and PC2 in the granular cultures (G1 and G2).

The loading map of the variables (individual LCFAs; see Figure 8.12) indicates that C16 and C12 are the most important by-products contributing to the variation explained by the two principal components. LCFAs shorter than LUA were not significant, the measured concentrations of CAA, for example, agreed with the finding of (Rinzema *et al.* (1994) that CAA was rapidly degraded without significant accumulation of  $\beta$ -oxidation intermediates. Generally, the lower degradation rate of LA in suspended culture was beneficial to the  $H_2$  yield in comparison to the larger degradation rate in granular culture.

The results suggest that low concentrations of LA, MA, and traces of LUA can direct electron equivalents towards  $H_2$  in suspended cultures and maintain a yield of more than  $2 \text{ mol } H_2 \cdot \text{mol}^{-1}$  glucose. Because such combination of acids was obtained from the degradation of LA over 20-25 days of incubation, more experimental work is required to verify whether a similar combination of externally added acid would results in increased  $H_2$  yield.

The similarities and differences in the profiles of the glucose fermentation by-products of the glucose controls and glucose plus LA-treated samples of the granular and flocculated cultures were shown using PCA. The glucose controls of the flocculated and granular cultures were different from each other, and each was different from its corresponding LA-treated samples. However, the LA-treated samples of the granular and flocculated cultures clustered with each other. The flocculated and granular cultures are from the same microbial sample and contain the same species. LA changed the composition of the microbial population (i.e. percentage of each species) which dominated each culture. LA is known to reduce differences between cultures obtained from different sources, so that the  $H_2$  yield is optimized to similar levels (reviewed in Chapter 1). In this study, the findings suggest that LA can improve the  $H_2$  yield from cultures obtained from the same source, depending on the structure of the culture (granular or flocculated).

PCA clearly established the shift in the composition of the bacterial community due to the addition of LA as well as the differences between the dominant species present in the flocculated and granular cultures. The LA layer adsorbed on the external surfaces of the granules may have protected the microorganisms in the core from the effects of LA and

from the increasing VFAs produced during glucose fermentation. The PCA score map indicates that the granular culture were close to the original untreated culture. The samples from the flocculated culture were different from each other, from the original untreated culture, and from the granular culture samples likely because different degrees of inhibition accompanied the presence of LA and the VFAs produced during its degradation. Population redundancy among glucose fermenters, differences in the kinetics of substrate degradation, and differences in species' survival capability under stressful conditions (exposure to LCFAs) created sufficient diversity to support the processes observed in flocculated cultures (Fernandez *et al.*, 1999).

The H<sub>2</sub> yields in this study are comparable to those reported previously for LA applied for shorter incubation periods (Chowdhury *et al.*, 2007). The results suggest that 2,000 mg L<sup>-1</sup> LA can support H<sub>2</sub> production from glucose for not less than one month with an average yield of 2.2 mol H<sub>2</sub>·mol<sup>-1</sup> glucose using flocculated cultures. Therefore, data from these studies provide experimental evidence for the hypothesis of Chowdhury *et al.* (2007) that the inhibitory effects caused by LA is not expected to vary significantly over an extended period.

## 8.5 CONCLUSIONS

The flocculated culture showed greater inhibition than the granular culture and diverted more electron equivalents towards H<sub>2</sub>, whereas the granular culture diverted electron equivalents to methane and other by-products. Flocculated culture samples produced more H<sub>2</sub> and less methane than granular culture samples. of A 63% maximum biological conversion efficiency of glucose to H<sub>2</sub> (the culture produced 63% of the maximum 4.0



mol H<sub>2</sub> mol glucose) was achieved using a flocculated culture which was incubated with LA for 25 days.

Methanogens did not adapt to the inhibitory effects of LCFAs over the various incubation periods tested (0 to 25 days). Degradation of LA by  $\beta$ -oxidation to PA, MA, LUA, and acetate was observed over the various incubation periods (0 to 25 days). PA was the principal by-product from LA degradation. The profiles of the LA degradation by-products and the rate of LA degradation differed for the flocculated culture and the granular culture derived from the same source (the original, untreated culture).

The flocculated culture samples differed from each other in their bacterial composition, whereas the granular culture samples were similar to the original untreated culture. *Bacteroides* dominated the granular culture whereas *Clostridium* and *Bacillus* sp. dominated the flocculated culture.

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## CHAPTER 9: SUMMARY AND CONCLUSIONS

### 9.1 SUMMARY

Mixed anaerobic cultures are available from many sources such as municipal and industrial wastewater treatment plants, compost, soil, and sediments. They are preferred over pure cultures for H<sub>2</sub> fermentation because they are readily available, easily maintained, and do not need sterilized conditions, thus they can use wastes as substrates. A sustainable and green H<sub>2</sub> production process requires environmentally friendly and renewable substrates, inocula, and inhibitors. Long chain fatty acids (LCFAs) are natural non-toxic environmentally friendly biodegradable organic inhibitors to methanogens and can be obtained as a waste stream from food processing plants. Polyunsaturated LCFAs such as linoleic (LA) and oleic acids (OA) increase the yields of H<sub>2</sub> in mixed culture dark fermentation. They inhibit the terminal metabolic step of methanogenesis and divert electron equivalents towards H<sub>2</sub> (Chowdhury *et al.*, 2007; Ray *et al.*, 2008). Because mixed cultures are considered a ‘black-box’, the dynamics of their microbial communities during H<sub>2</sub> fermentation are still not well understood.

Although LCFAs are reportedly superior to other pretreatments in H<sub>2</sub> production studies, their effects on cultures from different sources, cultures with different physical structures, the phenotypic diversity of microbial communities, and the kinetics of the various trophic groups in H<sub>2</sub>-producing mixed cultures were not assessed prior to this study.

This work examined, assessed, and quantified several parameters of mixed culture H<sub>2</sub> fermentation from glucose using LCFAs as inhibitors. A schematic description showing

the work in each chapter and how they integrate with each other is depicted in Figure 9.1. The integration of the characterization tests into the various experiments enabled studying the dynamic of LCFA inhibited  $H_2$  producing anaerobic mixed cultures.

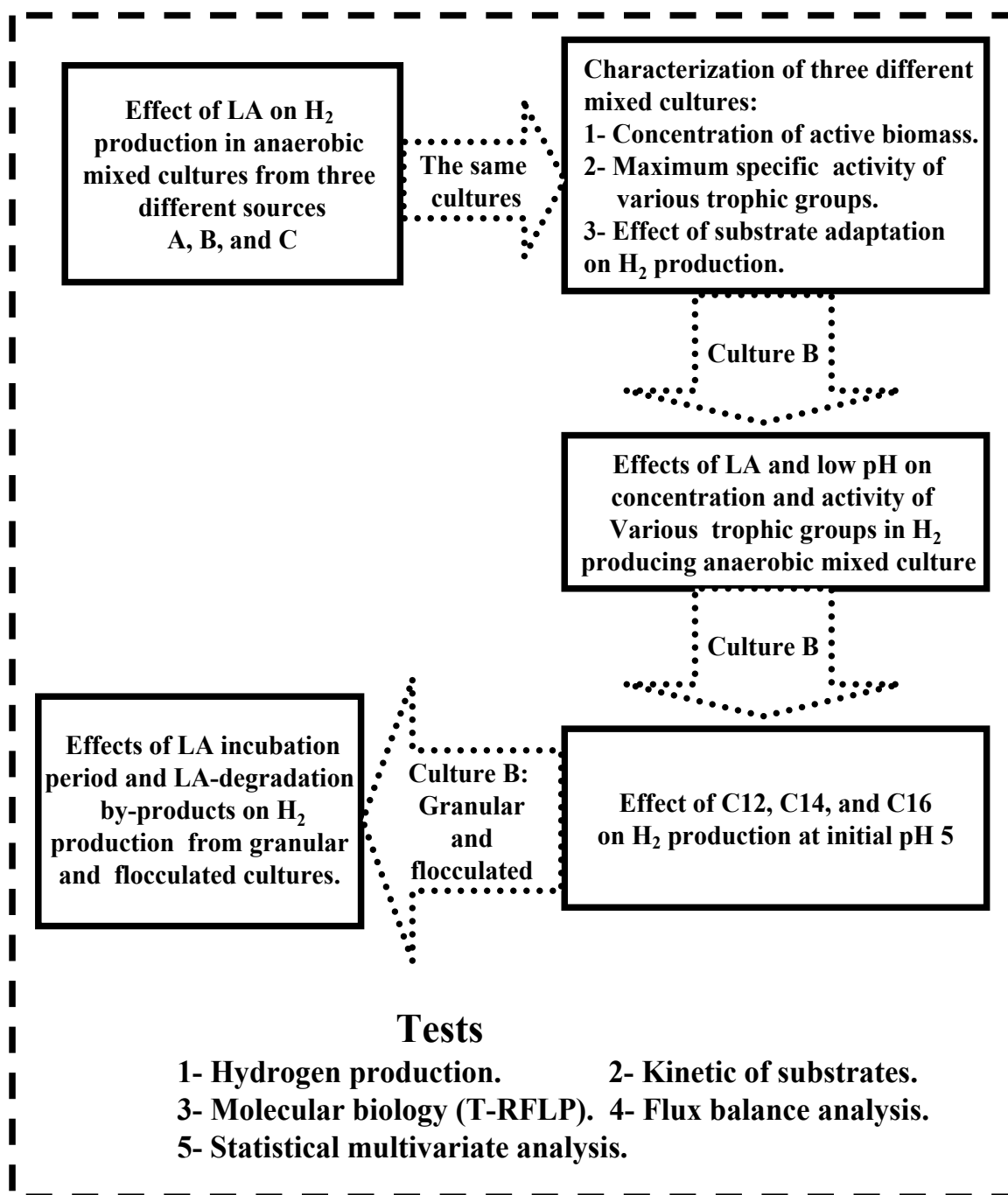


Figure 9.1 Schematic of the components of the research work.



The effects of LCFAs on H<sub>2</sub> production in three cultures from different sources, physical structure, and microbial communities were assessed in phase I. These three cultures were subjected to further characterization in phase II using kinetic and microbiological methods; the effects of long-term adaptation to glucose on the population and activity of various trophic groups in the cultures were quantified along with the overall effect on H<sub>2</sub> yield. Phase III evaluated the effects of low pH and the addition of LA on the population and activity of the various trophic groups in H<sub>2</sub>-producing mixed culture. T-RFLP analysis was conducted under the various incubation conditions to characterize and monitor the dynamics of the microbial community. In phase IV, the effects of LCFAs shorter than C18:0 (i.e., PA, MA, LUA, and a mixture of 50:50 PA:MA) on H<sub>2</sub> production were evaluated; the microbial communities in the culture incubated with the various LCFAs were analyzed using T-RFLP. The final phase of this research (phase V) examined the effects of long incubation periods (0-25 days) and LA degradation by-products on the H<sub>2</sub> yield of granular and suspended cultures with the same origin. All experiments were conducted in 160 mL serum batch reactors with 50 mL working volume and incubated at 37±2°C. The VSS was 2,000 mg L<sup>-1</sup> and all H<sub>2</sub> production bottles were fed glucose (5,000 mg L<sup>-1</sup>). Reactors treated with LCFAs received 7.13 mM.

In phase I, the effects of LCFAs on H<sub>2</sub> production in three cultures from different sources, physical structure, and microbial communities were assessed. The cultures used in this study were as follows: Culture (A) - granular methanogenic culture from a UASB treating wastewater from a brewery; Culture (B) - granular methanogenic culture from UASB from an ethanol manufacturing plant; and Culture (C) - suspended/flocculent

acidogenic culture from an anaerobic digester at a municipal wastewater treatment facility. The cultures differed in their origin, physical structure, and microbial composition.

H<sub>2</sub> yields obtained from the three LA-treated cultures were: 3.11±0.02 (A), 3.11±0.17 (B), and 3.13±0.14 (C) mol H<sub>2</sub>·mol<sup>-1</sup> glucose, whereas in the corresponding control cultures, the H<sub>2</sub> yields were: 1.61±0.12 (A), 2.80±0.48 (B), and 2.80±0.12 (C) mol H<sub>2</sub>·mol<sup>-1</sup> glucose. No significant differences were observed in H<sub>2</sub> yields from the three cultures examined under the stress condition of 2,000 mg L<sup>-1</sup> LA with an initial pH 5.0 even though the control cultures differed significantly from each other. Differences observed among the three cultures included the distribution of by-products and H<sub>2</sub>-consumption during the H<sub>2</sub> production experiment.

LA increased the H<sub>2</sub> yields in cultures A, B, and C by 93%, 11%, and 12%, respectively, and decreased the glucose initial degradation rates by 23% (A), 69% (B), and 37% (C). Post hoc pair wise comparisons (p<0.05, Tukey's test) indicated that initial glucose degradation rates in methanogenic cultures (A and B) were not significantly different from each other; however, both differed significantly from the glucose initial degradation rate in the acidogenic culture (C).

An electron mass balance indicated that 26% of the electrons in glucose were recovered as H<sub>2</sub> (recovery efficiency of 78%) in LA-treated culture. LA increased the H<sub>2</sub> yield by minimizing H<sub>2</sub> consumption by methanogens; however, the findings from this study indicate the need for more research into methods of controlling homoacetogenic H<sub>2</sub> consumption during H<sub>2</sub> fermentation.

The principal components analysis (PCA) of the data sets for H<sub>2</sub> yields and metabolic by-products on different days during the course of fermentation confirmed the effects induced by LA. Control samples from cultures A, B, and C were different from each other, and each control culture also differed from its corresponding LA-treated culture on the score maps of PCA. LA-treated cultures clustered together on the score maps; however, note this cluster was very distinct from the scattered points representing the control cultures.

Experimental data from these studies were modeled using kinetic and biochemical approaches. Kinetically, the modified Gompertz model described the progression of H<sub>2</sub> production in control and LA-treated cultures very well ( $R^2$  value between 0.94 and 0.98). However, the model was insensitive to H<sub>2</sub> consumption and does not account for or predict any loss in H<sub>2</sub> after the observed peak. The flux balanced analysis (FBA) explained the increased H<sub>2</sub> yields detected in LA-treated samples. The FBA model showed that LA increased the yield of H<sub>2</sub> by increasing the pool of Fd<sub>red</sub> available for the reduction of protons to H<sub>2</sub> and by minimizing NADH<sub>2</sub> consumption during lactate and propionate formation. Moreover, the model provided an insight into H<sub>2</sub> consumption by homoacetogenic bacteria.

In phase II, the cultures (A, B, and C) examined in phase I were characterized microbiologically and kinetically. The objective was to assess the differences and similarities in the various trophic groups and the species comprising the microbial communities of the cultures before and after twelve months of adaptation to glucose. The percent biomass of the various active trophic groups and their metabolic activities were determined before and after adaptation.

In general, adaptation to glucose increased the biomass concentration of active H<sub>2</sub> producers and concurrently decreased the activity of H<sub>2</sub> consumers. The adaptation to glucose increased the biomass concentration of glucose degraders (H<sub>2</sub> producers) by 66%, 53%, and 16% in cultures A, B and C, respectively. The increase in acidogenic biomass concentration contributed to the increase of the total active biomass. The latter increased in all three cultures by 54% (A), 95% (B), and 41% (C), respectively. On the other hand, the biomass concentration of the active H<sub>2</sub> consumers also increased after adaptation in culture A, B, and C (particularly in B); by 110% (A), 560% (B), and 145% (C), respectively. However, H<sub>2</sub> consuming activity decreased after adaptation by 72% (A), 55% (B), and 53% (C), respectively. Generally, specific activities of all trophic groups except glucose degraders decreased after adaptation.

The combined effects of the increase in the percentage of the biomass of active acidogens, the increase in acidogenic activity, and the decrease in H<sub>2</sub> consuming activity resulted in increased H<sub>2</sub> yields. This is in agreement with previously reported adaptation-induced changes, which included elevated levels of cellular transporters and enzymes, changes on the cell surface, and metabolic changes that lead to increased acid production (Padan *et al.*, 2005). The H<sub>2</sub> yields from adapted and non-adapted cultures were significantly different; this finding was supported by the outcome of the PCA of the H<sub>2</sub> yield data set, which also indicated a difference between glucose-adapted and non-adapted cultures and confirmed the positive impact of long-term glucose-adaptation on H<sub>2</sub> yield.

These results indicate that the changes induced by adaptation to glucose in the percent biomass concentration of the various active trophic groups were not correlated with

changes in their specific activities, except for glucose degraders. Statistical analysis of the kinetic data from adapted and non-adapted cultures did not establish a systematic relationship in terms of the changes in the specific activities. This is likely due to the existence of metabolic and functional redundancy in the composition of the cultures, which was confirmed by the T-RFLP analysis. Moreover, while the activity of a particular species may change with environmental conditions, in general, the culture will maintain sufficient substrate-consuming activity. This might be due to the nature of substrate feeding frequency as well as the mixotrophic nature of species detected in mixed culture. However, the PCA revealed that cultures A, B, and C were kinetically different from each other both before and after glucose-adaptation. The differences between the adapted and non-adapted cultures are evident for each of culture A, B, and C on the score map of their projections on the principal components' axes. Projections for adapted cultures are located far from the corresponding non-adapted cultures. No clustering was observed; however, some similarity within the domain of adapted cultures or the domain of non-adapted cultures was not established by the PCA.

The long-term adaptation to glucose reduced the diversity of cultures A, B, and C as indicated by the Shannon diversity index (Table 9.1). Glucose-adaptation affected the diversity of cultures A and C more than culture B; fewer dominating bands were detected in cultures A and C after adaptation to glucose when compared to the non-adapted control cultures.

Table 9.1 Shannon diversity index for cultures A, B, and C before and after adaptation to glucose for 12 months.

<b>Culture</b>	<b>A</b>	<b>B</b>	<b>C</b>
<b>Adapted</b>	1.945±0.15	3.54±0.05	2.88±0.05
<b>Non-adapted</b>	3.32±0.04	3.64±0.08	3.21±0.18

Notably, *Clostridium botulinum* was dominant in two cultures and while *Clostridiiforme* was present in one culture after glucose-adaptation. *Brevibacillus* and *Syntrophobacter* species disappeared from all cultures after adaptation. *Bacteroides*, *Parabacteroides*, *Fusobacterium*, *Syntrophobacter*, and *Thermoanaerobacter* were eliminated from at least two cultures after adaptation. The PCA score maps of the T-RFLP profiles showed distinct differences between cultures A, B, and C before and after adaptation. The glucose-adapted cultures were clustered together whereas the non-adapted cultures were scattered across the score map.

Species with fermentative metabolism such as *Pelobacter propionicus* and *Desulfobulbus propionicus* ferment lactate to acetate and propionate (Schink 1984; Stams *et al.*, 1984; Samain *et al.*, 1982). Furthermore, homoacetogens can ferment lactate solely to acetate (Braun *et al.*, 1981; Andreesen *et al.*, 1970). The higher maximum specific growth rates of fermentative bacteria in comparison with syntrophs (Stams, 1994) and the experimental observation that acetate is the only liquid by-product detected in lactate-degradation assays support the explanation that fermentative activity attributes to higher maximum specific lactate utilization rates.

The molecular 16S RNA microbiological analysis revealed changes in the composition of the cultures due to glucose-adaptation. This analysis also is supported by previous findings which indicated changes in the composition of complex anaerobic microbial communities over time (Halenbeck and Ghosh, 2009). T-RFLP profiles revealed some specific phylotypes for each culture in addition to the common phylotypes present in all cultures. *Bacteroides*, *Clostridia*, *Eubacteria*, and *Lactobacillus* are known to be dominant in anaerobic bioreactors (Toerien and Hattingh, 1969). According to Britz *et al.*

(1994), *Clostridia*, *Bacillus*, *Entrobacter*, *Fusobacterium*, and *Klebsiella* are H<sub>2</sub>-producing acidogens which are commonly found in anaerobic bioreactors. All these species are capable of fermenting glucose and producing different quantities of H<sub>2</sub> and VFAs. Elimination of *Bacteroides*, *Parabacteroides*, and *Lactobacillus* after glucose-adaptation may have contributed to the increased H<sub>2</sub> production in the glucose-adapted cultures. Comparing the T-RFLP profiles of the bacterial populations using PCA demonstrated that adaptation to glucose created different bacterial communities.

The maximum degradation rate for each substrate by mixed culture depends on the concentration and activity of particular trophic group catabolising the substrate (Aguiar *et al.*, 1995). Differences in the maximum specific substrate utilization rates of different cultures under the same environmental conditions represent differences in the species comprising bacterial populations or their growth conditions. The disappearance of slow-growing acetogenic species such as *Fusobacterium* and *Syntrophobacter* (e.g., *Syntrophus aciditrophicus*) from cultures A, B, and C may explain the observed decrease in the maximum specific utilization rates of butyrate and propionate after glucose-adaptation. The metabolism of these compounds by H<sub>2</sub>-producing acetogenic bacteria depends on the activity of H<sub>2</sub>-consuming partners. Notice the association between the decrease in the maximum specific utilization rate of H<sub>2</sub> and those of propionate, butyrate, and lactate. These results support previous findings that the rate of VFA-degradation is proportional to H<sub>2</sub>-consuming activity (Voolapalli and Stuckey, 1999).

Hydrogen-consuming bacteria were detected in cultures A, B, and C, including *Nocardia*, *Paracoccus*, *Eubacteria*, *Deslfovibrio* and *Desulfobacter*. The reduction in the dominance of sulfate-reducing bacteria (SRB) after glucose-adaptation might partially

account for the decrease in H<sub>2</sub>-consumption activity observed in all cultures after adaptation to glucose. SRBs declined, but were not eliminated and their fermentative growth with pyruvate and fumarate is faster than that of *Syntrophobacter* species (Kuever *et al.*, 1986). Furthermore, *Syntrophobacters* species are unlikely to dominate a culture containing VFAs such as propionate because of their slow growth rates (Kuever *et al.*, 1986).

Redundancy in the metabolic functions of bacteria in mixed cultures makes it difficult to correlate specific activities to the population (or percentage of the biomass) of the specific trophic group. In anaerobic fermentative systems, microorganisms may have similar growth rates and yields and many can catalyze several shared fermentative reactions at the same time (Rodriguez *et al.*, 2006). Examples of functional redundancy in mixed anaerobic cultures were discussed in section 5.5 with an attempt to correlate changes in the biomass concentrations and specific activities due to adaptation. To date, there were no tools or methods available to quantify concurrent metabolic activities of the various species in a mixed culture sample.

Facultative anaerobic bacteria either were unaffected or became dominant after glucose-adaptation. Although their greater abundance does not necessarily imply higher physiological activity than that of the strict anaerobes, it would be surprising if these high abundance levels for facultative anaerobes do not contribute significantly to total physiological activity under the incubation conditions. Oh *et al.* (2003) found evidence that 15 aerobic species (such as *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp.) from a suspended culture produced between 1.02-1.22 mol H<sub>2</sub>·mol<sup>-1</sup> glucose under anaerobic conditions (Oh *et al.*, 2003).



Elimination of some species from one culture and their dominance in another after glucose-adaptation might reflect the absence or existence of microbial interactions among these species and other microorganisms in that specific culture. These interactions could enable the dominance of a microorganism; otherwise, that species would disappear from the culture during adaption.

The combination of kinetic, microbiological, and principal components analyses elucidated the effects of long-term adaptation to glucose on the three cultures. A kinetic approach (determination of the biomass concentrations of individual trophic groups and their metabolic activities) was used to monitor and detect potential unbalanced situations among the different bacterial species in H<sub>2</sub> production processes.

In phase III, experiments were conducted to quantify the effects of lowering the initial pH from 7.6 to 5.0 and then adding 2,000 mg L<sup>-1</sup> LA on the biomass concentration and specific activities of active acidogens (glucose-degraders), acetogens (lactate-, propionate-, and butyrate-degraders), acetoclastic bacteria (acetate-degraders); and hydrogenotrophic methanogens and homoacetogenic bacteria (H<sub>2</sub>-degraders). Notice that pH 5.0 plus 2,000 mg L<sup>-1</sup> LA is the optimum condition for H<sub>2</sub> production. The specific activities of all trophic groups, except acetate- and H<sub>2</sub>-consumers, increased after lowering the initial pH from 7.6 to 5.0 whereas the biomass concentration of all active trophic groups decreased. Post hoc analyses (two-tailed t-tests;  $p < 0.05$ ) revealed that the specific activities and percentages of biomass concentration of the active trophic groups of glucose-, lactate-, acetate- and butyrate-degrading microorganisms in samples with initial pH 7.6 were significantly different from those of the same culture with initial pH 5.0.

After adding LA, the acidogenic population lost 47% of their maximum glucose specific utilization rate and 16% of their active biomass concentration. In comparison, the maximum lactate specific utilization rate decreased by 97% and the percent lactate degraders in the active biomass decreased by 92%.

Lowering the initial pH from 7.6 to 5.0 decreased the H<sub>2</sub> maximum specific utilization rate by 24 % (relative to the rate at pH 7.6). The addition of LA (2000 mg L<sup>-1</sup>) decreased the H<sub>2</sub> maximum specific utilization rate by 93% (relative to the rate at pH 5.0) while the reduction was 99% relative to the rate at pH 7.6. Hence, lowering the pH was responsible for only 24% of the total reduction while the addition of LA accounted for 75% of the total reduction in the H<sub>2</sub> maximum specific utilization rate. Furthermore, post hoc analyses (Tukey's test; p<0.05) revealed that the maximum H<sub>2</sub> specific utilization rates of the cultures at pH 7.6 and pH 5.0 were significantly different from each other, and they both were significantly different from the rate at pH 5.0 after the addition of LA (Table 9.2).

Table 9.2 Hydrogen consumption rates in mixed anaerobic culture at pH 7.6, 5.0, and pH 5.0 plus LA at 37°C.

<b>pH</b>	<b>LA (mg L<sup>-1</sup>)</b>	<b>H<sub>2</sub> consumption rate (day<sup>-1</sup>)</b>
7.6	0	20.56±0.97 <sup>a</sup>
5.0	0	15.62±2.27 <sup>b</sup>
5.0	2,000	1.08±0.21 <sup>c</sup>

Note: means with similar superscripts are statistically similar. Means with different superscripts are significantly different (p<0.05).

Although LA decreased the activity of H<sub>2</sub>-consuming microorganisms by 99%, the biomass concentration of the active H<sub>2</sub>-degraders increased by 72% in cultures incubated at initial pH 5.0 with LA compared to pH 5.0 alone. The T-RFLP profiles of the

microbial community during H<sub>2</sub> consumption experiments showed the presence of non-methanogenic H<sub>2</sub>-consuming microorganisms. For example, species such as *Moorella thermoacetica* (Drake and Daniel, 2004; Gößner *et al.*, 2008), *Eubacterium*, *Acetobacterium*, and *Syntrophobacter* (Drake, 1994) are known homoacetogens. In comparison to acetogenesis, these microorganisms derive more thermodynamic metabolic efficiency from consuming H<sub>2</sub> (homoacetogenesis) than from producing it when they grow on multiple carbon compounds (Marchaim, 1992). The greater diversity of bacteria observed in LA-treated samples in the H<sub>2</sub> consumption assay compared to the control samples (received H<sub>2</sub> without LA) is likely because H<sub>2</sub> was left available as an excellent electron donor to homoacetogens, sulfate- and iron-reducers, denitrifiers, and aromatic compound oxidizers after LA treatment inhibited the methanogens (Lee *et al.*, 2009).

T-RFLP profiles from the H<sub>2</sub> production studies showed that LA altered the profile of dominant species from *Bacteroides* sp. (22.8%); *Bacillus* sp. (19%); *Clostridium* (12%); *Acinetobacter*, and *Falvobacterium* sp. (6%); *Anaeroplasma abactoclasticum* (5%); *Eubacterium* (4 %); and *Rubrobacter* (4%) in the glucose control culture to *Clostridia* (35%); *Bacteroides*, *Flavobacteria*, and *Syntrophus* (28%); *Geobacillus* (10%); *Bacteroides* and *Actinobacillus* (4%); and *Citrobacter*, *Cytophaga*, *Enterobacter*, *Erwinia*, *E. coli*, and *Klebsiella*. Control cultures from the H<sub>2</sub> consumption studies (received only H<sub>2</sub>/CO<sub>2</sub>) were dominated by *Clostridium* (43%); *Prochlorococcus marinnus* (20%); *Victivallis vadensis* (17%); *Bacillus* (11%); and *Rubrobacter* and *Paennibacillus* (9%). LA-treated samples from the H<sub>2</sub> consumption experiment (received H<sub>2</sub>/CO<sub>2</sub> plus LA) were dominated by *Bacteroides* and *Rubrobacter* (42%); *E. coli* (14%); *Flavobacterium* (10%); *Actinobacillus* (9%); *Bacillus* (6%); *Citrobacter*, *Enterobacter*,

and *Flavobacterium* (5%); *Clostridium* (3%); *Propionibacterium* and *Thermoanaerobacter* (3%); *Butyrivibrio*, *Cytophaga fermentans*, and *Klebsiella* (3%); *Lactobacillus* (2%); *Parabacillus* (1%); and *Syntrophus* (1%). The total relative abundance of *Clostridia* in LA-treated culture from the H<sub>2</sub> consumption experiment was 11%. Therefore, LA decreased the abundance of *Clostridia* in H<sub>2</sub>-consuming samples and increased its H<sub>2</sub>-producing microbial capacity.

PCA of T-RFLP profiles showed distinct differences between the control and LA-treated cultures under the different conditions. LA-treated cultures from experiments of H<sub>2</sub> production, H<sub>2</sub> consumption, and lactate-, acetate-, propionate-, and butyrate-degradation behaved similarly and clustered together on the score maps of the principle components of the T-RFLP data.

The results suggest that species other than *Clostridia* also play an important role in H<sub>2</sub> consumption. In addition, species such as *Moorella thermoacetica* (Drake and Daniel, 2004; Gößner *et al.*, 2008), *Eubacterium*, *Acetobacterium*, *Syntrophobacter* (Drake, 1994) and some SRBs are known homoacetogens (see sections 2.2.3, 2.2.4 and Table 2.3).

Several bacterial genera capable of producing propionate include *Propionibacteria*, *Veillonella*, *Clostridium*, *Selenomonas*, *Megasphaera*, *Bacteriodes* and *Fusobacterium* (Border *et al.*, 1987). Although many of these non-sporulating organisms were detected in the LA-treated culture and their concentrations were higher than in the control cultures, no propionate accumulation was observed in the LA-treated cultures during the H<sub>2</sub> production experiment. Reasons for not detecting propionate in the control cultures are unclear but this could be related to conditions slowing or inhibiting the growth of

propionate producing microorganisms. Although propionate producers were detected in the LA-treated culture, LA inhibition could have resulted in propionate accumulation.

The flux balanced analysis (FBA) supports the findings of the kinetic study. The FBA model predicted that no homoacetogenic  $H_2$  consumption was observed in the LA-treated cultures; whereas a maximum of  $0.42 \text{ mol } H_2 \text{ mol}^{-1} \text{ glucose}$  (18% of the maximum yield) was consumed in the control cultures. Homoacetogenesis and solventogenesis consumed 58% and 43% of the  $H_2$  consumed in the control cultures, respectively. Production of isopropanol and propanol was expected because of the dominance of *Clostridium* sp., which can convert acetate, butyrate and  $H_2$  to propanol and butanol when the  $P_{H_2}$  is high (Jones and Woods, 1986). The biochemical and microbial conditions causing the shift to solventogenesis are discussed in section 6.5.

Other species which were present at high levels in LA-treated cultures included *Eubacterim*, *Bacillus*, *Geobacter*, *Thermoanaerobacterium*, and *Butyrivirio*. The heterogeneous spectrum and abundance of the dominant microorganisms observed in LA-treated samples distinguishes this method from other pretreatment procedures that produce homogeneous cultures with fewer dominating bands. An extensive comparison of the microbial diversities detected in the present studies with those reported previously is presented in Table 9.3. Notice LA and PA maintained greater diversities in comparison with other pretreatments.

Gram-positive bacteria are more susceptible to LCFAs inhibition than Gram-negative bacteria (Rinzema, 1988). Evidence from this and other studies; however, suggest that some Gram-positive bacteria can survive LCFAs inhibition. For example, *Butyrivibrio fibrisolvens* was detected in LA-treated cultures incubated with acetate and butyrate, and

Table 9.3 Microbial diversities for mixed cultures producing H<sub>2</sub> by dark fermentation.

Inocula	Treatment	Species detected	Yield <sup>a</sup>	Reference
Anaerobic culture	LA (C18:2)	<i>Bacteroides</i> , <i>Flavobacteria</i> , and <i>Syntrophus</i> (27.89%); <i>Clostridia</i> (10.07%); <i>Clostridia</i> and <i>Geobacillus</i> (10.20%); <i>Clostridia</i> (15.10%); <i>Bacteroides</i> and <i>Actinobacillus</i> (4.08%); <i>Citrobacter</i> , <i>Cytophaga</i> , <i>Enterobacter</i> , <i>Erwinia</i> , <i>E. coli</i> , and <i>Klebsiella</i> (3.40%)	3.1	This study
Anaerobic culture	PA (C16:0)	<i>Bacillus</i> and <i>Clostridium</i> (34.14%); <i>Bacteroides</i> , <i>Bacillus</i> , and <i>Bifidobacterium sp.</i> (13.9%); <i>Geobacter lovely</i> (12.14%) <i>Bacteroides</i> (10.46%); and <i>Thermoanaerovibrio</i> (6.82%)	2.53	This study
Soil	HT	<i>Clostridium</i>	1.93	Ravindran <i>et al.</i> 2010
Municipal wastewater sludge	Repeated-aeration	<i>Ethanoligenens</i> , <i>Enterobacter</i> , and <i>Bacteroides</i>	1.96	Ren <i>et al.</i> 2008
	Acid	<i>Propionibacterium propionicus</i> <i>Clostridium</i>	0.45	
	Alkaline	<i>Bacteroides</i> , <i>Clostridium</i>	1.17	
	HT	<i>Clostridium</i>	1.65	
Anaerobically digested municipal biosolids	HT	<i>Clostridium</i> and <i>Bacillus</i>	1.53 <sup>b</sup>	Sung <i>et al.</i> 2002
domestic wastewater sludge	HT	<i>Clostridia</i> (68%), <i>Klebsiella</i> , and <i>Streptococcus sp.</i>	1.45	Hung <i>et al.</i> 2007
Anaerobic digested sludge	HT	<i>Thermoanaerobacterium</i>	1.16	O-Thong <i>et al.</i> 2009
	Load-shock with POME	<i>Thermoanaerobacterium</i>	1.96	
	Base	<i>Clostridium</i>	0.51	
	Acid	<i>Clostridium</i>	0.65	
	BESA	<i>Bacillus</i>	1.01	
Thermophilic anaerobic enrichment from compost	No	<i>Thermoanaerobacterium</i> , <i>Clostridia</i> , <i>Sulfobacillus disulfidooxidans</i>	2.0	Ueno <i>et al.</i> 2001
Domestic sewage sludge	No	<i>Bacteroidetes</i> , <i>Actinobacteri</i> , <i>Ethanoligenens</i> , <i>Clostridia</i>	-	Xing <i>et al.</i> 2008
H <sub>2</sub> producing thermophilic trickling biofilter reactor	No	<i>Clostridia</i> , <i>Bacilli</i> , <i>Thermoanaerobacterium</i>	1.11	Ahn <i>et al.</i> 2005
Municipal anaerobic sludge	No	<i>Clostridia</i> (64.6%), <i>Entrobacteria</i> (18.8%), and <i>Streptococcus</i> (3.1%)	1.91	Fang and Liu, 2002b
Municipal granular anaerobic sludge	No	<i>Clostridia</i> (69.1%), <i>Sporolactobacillus</i> (13.5%), and <i>Papillibacter</i> (5.8%)	2.1	Fang <i>et al.</i> 2002a

<sup>a</sup> (mol H<sub>2</sub>·mol<sup>-1</sup> glucose). <sup>b</sup> (mol H<sub>2</sub>·mol<sup>-1</sup> of sucrose). HT = heat-treatment.

POME = palm oil mill effluent.

also in control cultures containing acetate and propionate. Although *B. fibrisolvens* is classified as gram-negative, Cheng and Costerton (1977) observed, under electron microscopy, that it has a gram-positive-type cell wall. The observed dominance of *Clostridia*, *Bacillus*, and other Gram-positive species contradicts previous views.

In phase IV experiments were conducted to evaluate the impact of palmitic (PA, C16:0), myristic (MA, C14:0), lauric (LUA, C12:0) and a mixture of 50:50 palmitic:myristic (MA:PA) acids on H<sub>2</sub> yield from glucose in mixed culture at 37°C with an initial pH 5.0. The molar concentration of each acid was selected on a basis carbon basis equivalent to 2,000 mg L<sup>-1</sup> of linoleic acid (LA). The purpose of this phase was to diversify the pool of the LCFAs that could be used in biohydrogen production. The impact of such finding could decrease disposal cost and by producing an energy source from dairy effluents. This technology could be important for food processors in the dairy and fried food sectors.

The culture treated with PA produced the highest H<sub>2</sub> yield in comparison to the cultures treated with LUA, MA and MA:PA; however, it was less than the yields measured in LA-treated cultures in Phase I and II (Figure 9.1). The maximum yield obtained in PA-treated culture is comparable to the yield reported for heat-treated inoculum [2.8 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] (van Ginkel and Logan, 2005). Note this yield is greater than those previously reported at low pH. Fang and Liu (2002) reported 2.1 mol H<sub>2</sub>·mol<sup>-1</sup> glucose at pH 5.5, whereas Khanal *et al.* (2004) reported a maximum yield of 1.7 mol H<sub>2</sub>·mol<sup>-1</sup> glucose in a pH range of 4.5 to 6.5.

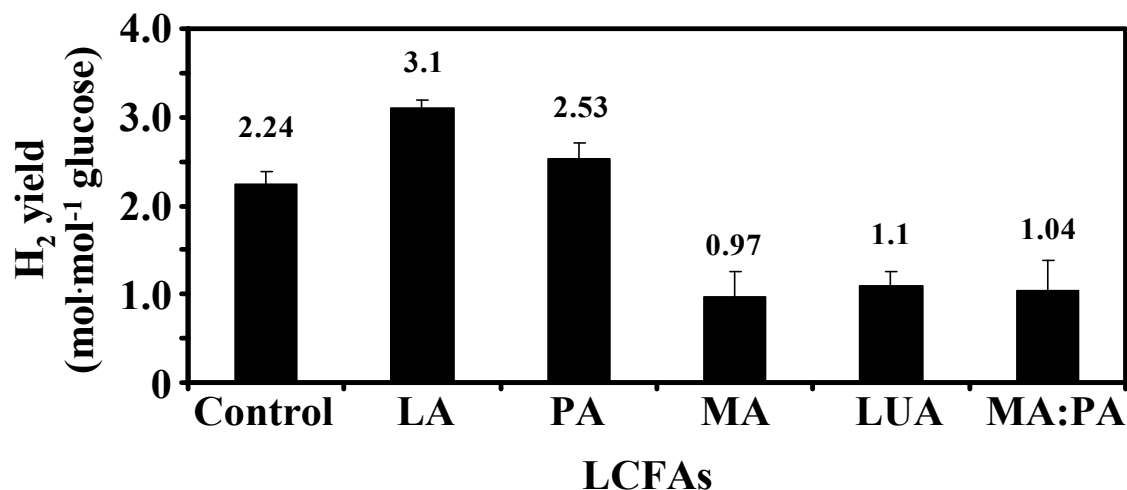


Figure 9.2 Maximum H<sub>2</sub> yields in control and cultures treated with lauric (LUA), myristic (MA), palmitic (PA), myristic:palmitic (MA:PA), and linoleic acids (LA). (MA:PA = mixture of 50:50 myristic:palmitic on a molar basis).

A comparison of the results from this study with previous studies that used LCFAs in H<sub>2</sub> production showed that the H<sub>2</sub> yield achieved in the presence of PA [2.53 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] is greater than the yield achieved with oleic acid [ (C18:1) 2.2 mol H<sub>2</sub>·mol<sup>-1</sup> glucose] (Sharma, 2008; Gurukar, 2005) and is comparable or slightly less than that achieved in other studies with LA [(C18:2); 2.4±0.11, 2.7±0.06, and 2.8±0.12 mol H<sub>2</sub>·mol<sup>-1</sup> glucose; (Ray *et al.*, 2008; Gurukar, 2005; Sharma, 2008)].

In comparison to other cultures, LA- and PA-treated cultures did not show a lag phase during H<sub>2</sub> production after both the first and the second glucose injections, whereas after the first glucose injection, a 24 h lag phase was observed in the controls and cultures fed LUA-, MA-, and MA:PA. The H<sub>2</sub> yields in LUA-, MA-, and MA:PA- treated cultures were statistically similar, but differed significantly from the yields in both the control and PA-treated culture. Notice the H<sub>2</sub> yields of the control and PA-treated cultures did not differ significantly from each other. Hence, PA is distinct from LUA, MA, and MA:PA in how it behaves on different microbial populations during H<sub>2</sub> production. The differences



in H<sub>2</sub> yield among the cultures treated with various LCFAs reflect the response of the acidogenic and hydrogenotrophic groups to specific LCFAs. PA was less harmful to acidogenic H<sub>2</sub> producers in comparison with the LCFAs shorter than C16 tested and this is indicated by the relative effect of each of the LCFAs on glucose degradation.

In comparison to the rates seen in control cultures, the various LCFAs decreased the glucose initial degradation rates by 88% (LUA) > 68% (PA:MA) > 66% (MA) > 56% (PA). The initial glucose degradation rates for LCFA-treated cultures were significantly different from that of the corresponding control; however, MA-, PA-, and MA:PA-treated cultures were not significantly different from each other.

PCA of the data set for by-products indicated differences among the LCFA-treated cultures. The responses of the control and PA-treated culture were mapped close to each other, but the MA- and LUA-treated cultures were scattered far from the control and PA-treated cultures on the score map. This strongly suggests that they differed in their microbial population composition. MA:PA-treated culture was closer (so more similar) to the PA- than the MA-treated culture.

PA-treated cultures directed higher percentages of electron equivalents to acetate and butyrate in comparison to the other LCFAs examined. Conversely, the LUA-treated culture produced increasing concentrations of more reduced products such as lactate, isopropanol, and ethanol. The by-products profile in LUA-treated culture was far from ideal for H<sub>2</sub> production. The results suggest that PA influenced bacterial metabolic activities, which resulted in acetate-butyrate fermentation and created physiological and physico-chemical conditions under which the microorganisms produced the maximum H<sub>2</sub> yield.

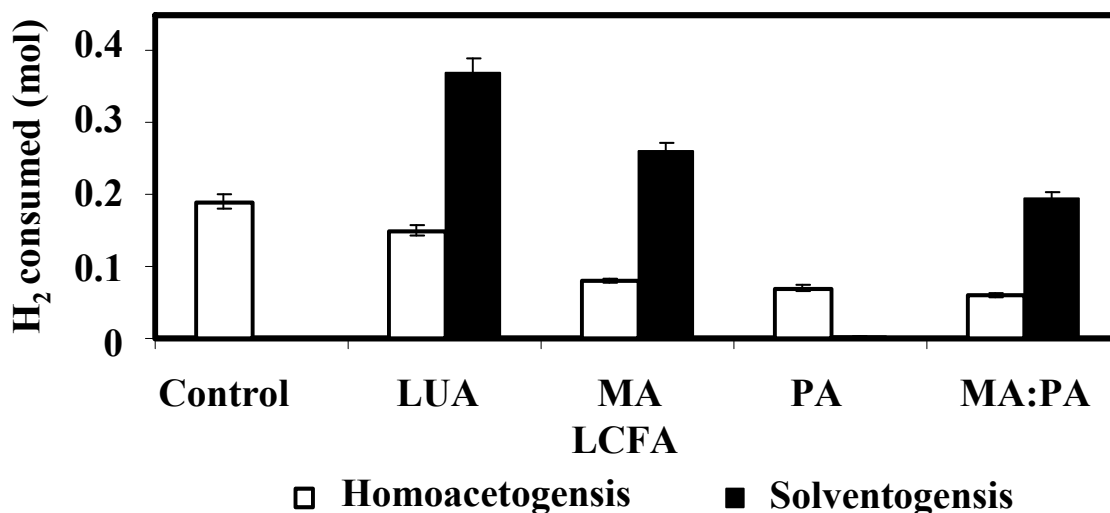


Figure 9.3 Homoacetogenic and solventogenic H<sub>2</sub> consumption in control and LCFA (LUA, MA, PA and MA:PA) treated cultures. (MA:PA = mixture of 50:50 MA:PA on a molar basis).

The FBA showed that homoacetogenesis and solventogenesis were the major H<sub>2</sub> sinks; (Figure 9.2). The activity of homoacetogenesis as a function of the LCFA added was as follows: Control > LUA > MA > PA > MA:PA whereas the ranking for solventogenesis was as follows: LUA > MA > MA:PA > PA > Control. The control and LCFA-treated cultures contained various concentrations of *Eubacteria*. with some species of different homoacetogens. The relative abundance (percentage) of *Eubacteria* was 28% (control) > 1.4% (LUA) > 0.2% (MA:PA) > 0.00% (PA). In addition, the dominant band in MA-treated culture included the Gram-positive bacteria homoacetogen *Acetobacteria*(Schink and Bomar, 1991). Only the *Acetobacteria* genera was identified in this work. Rinzema (1988) claimed that gram positive organisms are susceptible to LCFA attack; however, in this study, *Acetobacteria* was identified in cultures treated with MA. This observation could be related to the activity of homoacetogens as a function of the LCFA type and concentration.

The T-RFLP profiles revealed that *Thermoanaerovibrio acidaminovorans* dominated the control culture with an abundance of 60% followed by *Geobacillus* and *Eubacteria* (28%), while *Clostridia* formed less than 1% of the biomass. Treating the culture with LCFAs (MA, MA:PA, or LUA) increased the abundance of *Clostridia* and *Bacillus* to 87% (LUA) > 67% (MA) > 75% (MA:PA) > 48% (PA). However, although PA-treatment increased the relative abundance of *Clostridia* and *Bacillus* the least (to 48 %) and decreased *Thermoanaerovibrio acidaminovorans* to 7%, the abundance of other species such as *Geobacter lovely* and *Bacteroides* increased to 12 % and 10 %, respectively. A discussion of the potential role of *Geobacter* in converting acetate to H<sub>2</sub> is presented in section 6.5. Generally, PA-treated culture showed a more diverse bacterial population than the cultures treated with LUA, MA, or MA:PA.

The experiments in phase V assessed the impact of varying incubation periods (5 to 30 days at 37°C with an initial pH 5.0) and the by-products of LA degradation on H<sub>2</sub> production in granular and flocculated cultures from the same origin. Hydrogen and liquid by-products of glucose fermentation, LA degradation by-products, and the diversity of the microbial communities were monitored.

Generally, suspended culture treated with LA produced higher H<sub>2</sub> yields than the granular culture for all incubation periods (Table 9.4); an average H<sub>2</sub> yield of 2.2 mol

Table 9.4 Maximum H<sub>2</sub> yield in control and LA-treated granular and flocculated culture incubated for 0 to 30 days. (Values shown are mean ± standard deviation for triplicate samples).

<b>H<sub>2</sub> yield (mol H<sub>2</sub>·mol<sup>-1</sup> glucose)</b>			
<b><u>Granular culture</u></b>		<b><u>Suspended culture</u></b>	
<b><u>Control</u></b>	<b><u>LA (2,000 mg L<sup>-1</sup>)</u></b>	<b><u>Control</u></b>	<b><u>LA (2,000 mg L<sup>-1</sup>)</u></b>
<b>0.68±0.28</b>	<b>1.93±0.15</b>	<b>1.79±0.35</b>	<b>2.52±0.20</b>

$\text{H}_2 \cdot \text{mol}^{-1}$  was maintained for 30 days in LA-treated suspended culture. The smallest  $\text{H}_2$  yields observed in LA-treated culture was  $1.06 \pm 0.08$  and  $1.60 \pm 0.41 \text{ mol H}_2 \cdot \text{mol}^{-1}$  glucose in granular and flocculated culture incubated for 0 and 10 days, respectively.

Post hoc pairwise comparisons (Tukey's test;  $p < 0.05$ ) revealed that the maximum  $\text{H}_2$  yield in four out of six pairs (granular vs. flocculated culture) of LA-treated samples were significantly different. Hydrogen yields in glucose control containing granular cultures were significantly different from the yields in their corresponding LA-treated samples for all incubation periods under consideration. In flocculated culture, however, the  $\text{H}_2$  yields in 50% of the glucose control samples were significantly different from the  $\text{H}_2$  yields in LA-treated samples.

Glucose control granular cultures showed significant  $\text{H}_2$  consumption one day after the glucose injection. This trend continued throughout the experiment and no  $\text{H}_2$  was detected 5 days after injecting glucose. In comparison, 80% of the peak  $\text{H}_2$  levels produced in flocculated cultures treated with LA remained for 5 days after injecting glucose.

Methane was produced in control and glucose plus LA-treated cultures. Generally, granular culture controls produced more  $\text{CH}_4$  than suspended culture controls. LA inhibited  $\text{CH}_4$  production up to approximately 99% in comparison to glucose. Granular cultures produced increasing amounts of  $\text{CH}_4$  during incubation and the quantity of  $\text{CH}_4$  produced in LA-treated granular and flocculated cultures increased with the time of incubation. Nevertheless, the quantity observed in LA-treated samples was less than approximately  $200 \text{ } \mu\text{mol}$  of  $\text{CH}_4$  per bottle for granular culture and  $30 \text{ } \mu\text{mol}$  of  $\text{CH}_4$  per

bottle for the flocculated culture, whereas maximum levels of  $490 \pm 72$  and  $350 \pm 67$   $\mu\text{mol}$  of  $\text{CH}_4$  per bottle were observed in the respective glucose control samples.

Overall, LA affected the glucose initial degradation rate in the flocculated cultures more than in the granular cultures. After incubation with LA, the flocculated cultures lost approximately 50% of the acidogenic activity compared to control, whereas granular culture lost only 22%. Given that the granular and flocculated cultures used in this study were from the same origin, it appears that culture disintegration intensified the effects of LA in decreasing the acidogenic activity. Post hoc pairwise comparisons (Tukey's test;  $p < 0.05$ ) indicate, however, that the glucose initial degradation rates did not differ significantly for all other conditions tested in this phase.

The FBA analysis revealed that the order of  $\text{H}_2$  consumption (in mol of  $\text{H}_2$ ) was in the different cultures were as follows: granular + LA (1.17) > flocculated control (1.07) > granular control (0.63) > flocculated + LA (0.18). It was also clear that in the granular culture homoacetogenic  $\text{H}_2$  consumption became active only after methanogenesis was inhibited by LA. The control granular cultures consumed 0.63 mol of  $\text{H}_2$  (0.44 mol in methanogenesis and 0.19 mol in solventogenesis) compared to 1.17 mol of  $\text{H}_2$  in LA-treated samples (0.92 in homoacetogenesis and 0.25 mol in solventogenesis). In the control samples of flocculated cultures,  $\text{H}_2$  consumption (1.07 mol) proceeded via homoacetogenesis (0.88 mol) plus solventogenesis (0.18 mol) during propanol production. Notice, homoacetogenic  $\text{H}_2$  consumption in LA-treated suspended culture was zero.

Linoleic acid degraded in LA-control samples to undetectable levels within 20 days in the granular cultures to 25 days in the flocculated cultures at degradation rates of  $29 \pm 5$  and  $21 \pm 7 \mu\text{g LA} \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$ , respectively.

LA degradation by-products [PA (C16:0), MA (C14:0), and small quantities of LUA (C12)] were detected. The maximum levels of PA, MA and LUA reached  $890 \pm 76$  (PA),  $490 \pm 80$  (MA), and  $83 \pm 37$  (LUA)  $\text{mg L}^{-1}$ , respectively, in the LA flocculated culture controls, whereas in the LA granular controls, the maximum levels of PA, MA and LUA were  $1340 \pm 63$ ,  $290 \pm 13$ , and  $50 \pm 15$  (LUA)  $\text{mg L}^{-1}$ , respectively.. Peak LA level by-products were observed on different days over the duration of the experiment in the flocculated and granulated cultures: 1. Suspended culture – PA (day 25), MA (day 25), and LUA (day 10); 2. Granular culture – PA (day 25), MA (day 25), and LUA (day 20). Concentrations of PA decreased after 25 days in the flocculated cultures, but elevated levels were maintained in the granular culture.

LA degradation was observed in granular and flocculated cultures fed glucose and treated with LA. LA concentrations detected after 30 days of incubation were  $635 \pm 85$  and  $210 \pm 200 \text{ mg L}^{-1}$  in flocculated and granular cultures, respectively. The LA degradation rates in the control cultures were  $5.9 \pm 1.1$  and  $15.5 \pm 6.7 \mu\text{g LA} \cdot \text{mg VSS}^{-1} \cdot \text{day}^{-1}$  for the flocculated and granular cultures, respectively. Granular culture accumulated more PA than flocculated culture to a maximum level of  $780 \pm 120 \text{ mg L}^{-1}$  on day 20 and  $605 \pm 40 \text{ mg L}^{-1}$  in the flocculated culture on day 25. Low and transitory concentrations of stearic acid (SA, C18:0) were observed ( $130 \pm 75 \text{ mg L}^{-1}$ ) on day 20 in the granular culture. Similarly, low and transitory concentrations of OA were observed ( $280 \pm 320 \text{ mg L}^{-1}$ ) on day 10 in the flocculated culture. The maximum MA concentrations observed

were  $240 \pm 55 \text{ mg L}^{-1}$  on day 20 in granular cultures and  $940 \pm 430 \text{ mg L}^{-1}$  on day 5 in the flocculated cultures. Low concentrations of LUA were detected throughout all the different incubation periods. Peak levels of  $70 \pm 9 \text{ mg L}^{-1}$  LUA were detected in the flocculated culture while in the granular culture  $50 \pm 20 \text{ mg L}^{-1}$  were detected on day 5 and day 25, respectively.

PCA of the LA degradation by-products data set showed that the flocculated and the granular cultures behaved differently. The samples which were incubated over short incubation periods showed a great deal of variability to data from flocculated culture, whereas the samples with longer incubation periods showed more variability in the granular culture. On the score map of the PCA, data from both the flocculated and granular cultures were skewed in the direction of positive scores on the PC1 axis. However, with longer incubation periods, the observations from the flocculated culture were skewed in the opposite direction based on the location of observations from granular culture. The loading plot indicates that LA, PA, MA, and LUA were the most important LCFA by-products which contributed to the variation accounted for by the two principal components. PCA of the fermentation by-products data set showed that LA-treated granular and flocculated cultures were closer to each other in comparison to the controls. Moreover, the control cultures were located far from each other suggesting different responses under the low pH condition.

In addition to interspecies  $\text{H}_2$  transfer in granules, direct extracellular electron transfer from cell-to-cell through a c-type cytochrome was recently reported by Summers *et al.* (2010). These researchers demonstrated that electrically conductive microbial aggregates containing species of *Geobacter* are able to transfer electrons externally through

electrically conductive pili. The microbiological analysis using T-RFLP revealed that approximately 8% of the original culture was comprised of *Geobacter* which is able to grow chemoorganotrophically and can utilize substrates such as acetate, propionate, butyrate, and ethanol.

The detection of *Bacillus*, *Clostridium*, *Eubacterium*, *Propionibacterium*, and *Bacteroides* in the LA- granular and flocculated control cultures support the findings of Kemp *et al.* (1975), Verhulst *et al.* (1985), Fujimoto *et al.* (1993), and Hatamoto *et al.*, (2007). This research is reviewed in section 2.3.4. For example, species of *Clostridia* and *Butyrivibrio* sp. are able to hydrogenate unsaturated fatty acids (Sousa *et al.*, 2010).

Understanding of the dynamics of the microbial community under the various test conditions elucidates the experimental results. The greater diversity of fermentative and acidogenic bacteria (i.e. *Bacteroides* (52%); *Clostridium* (25%); *Bacillus*, *Brevibacillus*, and *Clostridium* (11%); and *Moorella thermoacetica* (5%)) observed in the flocculated culture fed glucose and treated with LA could account for the higher H<sub>2</sub> yield (2.52±0.20 mol H<sub>2</sub>·mol<sup>-1</sup> for culture incubated for 25 days with an initial pH 5 and 37°C). Under the experimental conditions under consideration, H<sub>2</sub> producers containing H<sub>2</sub>-evolving hydrogenase such as *Bacteroides* and *Clostridium* sp. were detected (Madigan *et al.*, 2000). Moreover, *Moorella thermoacetica*, a spore-forming acetogen which grows autotrophically on H<sub>2</sub>/CO<sub>2</sub>. Notice that FBA did not reveal homoacetogenic H<sub>2</sub> consumption in the flocculated cultures fed glucose and incubated with LA. Conversely, in granular culture fed glucose and treated with LA, the dominant species were *Bacillus thermoleovorans* (37%); *Eubacterium* (19%); *Clostridium*, *Butyrivibrio*, *Fusobacterium*, and *Gloeobacter* sp. (6%); and *Bacteroides* and *Falvobacterium* (4%). According to



Tanner *et al.* (1981), *Eubacterium* is a H<sub>2</sub>-oxidizing anaerobic bacterium and studies by Mechichi *et al.* (1998) have identified *Eubacterium aggregans* sp as a homoacetogens. The FBA predicted homoacetogenic H<sub>2</sub> consumption in the granular culture (G1) is supported by the T-RFLP data. Differences in the composition of the microbial communities could be a major reason which might explain the difference in H<sub>2</sub> yield between flocculated and granular cultures from the same culture source.

In the granular cultures, the percent *Bacteroides* decreased from 36% in the LA-control samples to 5% in the glucose-control samples, and to 2 % under the experimental condition (granular culture fed glucose and treated with LA). Concurrently, *Bacillus* increased from 0.0% in LA-control samples to 37 % in cultures fed glucose and treated with LA. Moreover, *Eubacterium* increased from 0.0% in glucose-control samples to 33% in LA-control samples, and to 19% in the culture fed both glucose and LA. The percentage of *Clostridium* sp. was almost the same (6%) in all conditions for granular culture. According to Nieman (1954), the levels of *Bacillus subtilis* in mixed cultures treated with Oleic (OA) and LA was transient and restricted to the early period of incubation.

The species dominating the LA-control flocculated cultures were different from those dominating the granular cultures. For example, *Bifidobacterium* (36%) and *Actinobacillus* and *Flavobacterim* (27%) dominated the LA-control granular cultures whereas in the in the LA-control flocculated cultures the dominant microorganisms were *Anaeroplasma* (12%), *Acinetobacter* (9%), *Alicyclobacillus* (8%), and *Corynebacterium* (7%) . Because these organisms are able to survive a stressful environment in the presence of elevated LA levels and low pH condition, they were likely played a role in degrading glucose as

well as H<sub>2</sub>, LA and the carbon fermentation by-products. To date some work has focused on understanding the role of the different microorganisms; however, a comprehensive understanding of their roles are lacking in the literature.

In this study, some components between Phase II and Phase V were integrated. In Phase II the long-term adaptation to substrate was assessed whereas, the possible adaptation of methanogens to LA during a month of incubation was examined in phase V. Both Phase II and V proved the stability of the culture performance in long-term studies. Phase III investigated the effects of LA on H<sub>2</sub> consumption and it was integrated with Phase I, II, and V to explain H<sub>2</sub> consumption during H<sub>2</sub> production from glucose fermentation. Phase III estimated the H<sub>2</sub> specific consumption rate under the stress conditions of LA plus low pH. Phase III identified a number of the H<sub>2</sub>-consuming microorganisms in the mixed culture which were also detected in other phases. Phase IV extended the experiment from Phase I and II by assessing the effects of different LCFAs. The results from Phase I, II, IV, and V indicated that mixed cultures from different sources could produce H<sub>2</sub> by inhibition with LA and potentially with PA. Generally, integrating work from the phases demonstrated that different sources of inhibited mixed anaerobic cultures can produce H<sub>2</sub>. The work also demonstrated that different LCFAs are able to inhibit mixed anaerobic cultures to a certain extent and subsequently produce H<sub>2</sub>. In addition, the cultures possess long term stability in terms of H<sub>2</sub> production even after long-term adaptation to both glucose and LCFAs.

## **9.2 CONCLUSIONS**

1. Based on this study, LCFAs can be used to manage and control the diversity and physiology of mixed cultures in H<sub>2</sub> dark fermentation for a period of not less than a

month. Because the LCFAs degrade very slowly, they can impose inhibition on a variety of microbial populations over long periods. Treatment with LA can overcome opposing factors in the mixed culture source, structure, and microbial communities which are favourable to increasing electron fluxes diversion to methane production. In this work, the H<sub>2</sub> yield reached 3.32±0.12 mol H<sub>2</sub>·mol<sup>-1</sup> glucose (conversion efficiency = 83%).

2. LCFAs maintain a great diversity of functionally redundant acidogenic microorganisms, which provides stability to the system's performance. Population redundancy among physiologically active glucose fermenters and differences in the capability of various species to survive under the stress conditions imposed by LCFAs and low pH supported process performance. Under conditions which LCFAs are able to impose microbial stress, maximizing bacterial diversity should be one of the engineering objectives in H<sub>2</sub> production because functional redundancy maintains and provides stability to process performance.
3. LCFA-treatment increased the abundance of *Clostridia*; however, they are not the only H<sub>2</sub> producers present in mixed culture:

A. LA-treated H<sub>2</sub>-producing cultures were dominated by *Clostridia* (35%); *Bacteroides*, *Flavobacteria*, and *Syntrophus* (28%); *Geobacillus* (10%); *Bacteroides* and *Actinobacillus* (4%); *Citrobacter*, *Cytophaga*, *Enterobacter*, *Erwinia*, *E. coli*, and *Klebsiella* (3%) while the control culture was dominated by *Bacteroides* (23%); *Bacillus* (19%); *Clostridium* (12%); *Acinetobacter*, and *Falvobacteria* (6%); *Anaeroplasma abactoclasticum* (5%); *Eubacteria* (4%); *Rubrobacter* (4%).

- B. Adding PA was able to increase the H<sub>2</sub> yield by approximately 13% (to 2.53±0.18 mol H<sub>2</sub>·mol<sup>-1</sup> glucose) compared to the control culture; however, LCFAs shorter than C16 were unable to attain this level of H<sub>2</sub> production. *Thermoanaerovibrio* (60%), *Geobacillus* and *Eubacteria* (28%) dominated the control culture while *Clostridia* comprised less than 1% of the biomass. Adding LCFAs were able to increase the abundance of *Clostridia* and *Bacillus* as follows: 48% (PA), 67% (MA), 75% (MA:PA), 87% (LUA). Furthermore, PA decreased the abundance of *Thermoanaerovibrio* to 7%, but increased *Geobacter* *lovely* plus *Bacteroides* to 12 and 10%, respectively. These changes show greater diversity than the other LCFAs-treated cultures. Similarly, LA increased the percent biomass of *Clostridia* in the glucose-, acetate-, propionate-, and butyrate-fed cultures, whereas it decreased the percent in the H<sub>2</sub>- and lactate-fed cultures.
4. Long-term adaptation to glucose has a positive impact on H<sub>2</sub> yield and a negative impact on H<sub>2</sub> consumption. Elimination of *Bacteroides*, *Parabacteroides*, and *Lactobacillus* and the reduction in the abundance of *Deslfovibrio* and *Desulfobacter* after adaptation to glucose partially accounts for the decrease in H<sub>2</sub>-consumption activity in cultures adapted to glucose for 12 months. Glucose adaptation could likely contribute to the increased H<sub>2</sub> yield observed in the glucose-adapted cultures. Disappearance of acetogenic species such as *Fusobacterium* and *Syntrophobacter* (e.g., *Syntrophus aciditrophicus*) from the cultures after glucose-adaptation explains the decrease observed in the maximum specific utilization rates of butyrate and propionate, which was associated with attenuation of the maximum specific

- utilization rate of H<sub>2</sub>. Sulfate-reducers such as *Deslfovibrio* declined, but were not completely eliminated. Facultative anaerobes such as *Eubacterium*, *Entrobacter*, and *Klebsiella* either were unaffected or became dominant after glucose-adaptation.
5. Homoacetogenic species other than *Clostridia* can play a significant role in H<sub>2</sub> consumption. Homoacetogens which were detected included *Moorella thermoaceticum*, *Eubacteria*, *Acetobacteria*, and *Syntrophobacter*, *C. Nocardia*, *Paracoccus*, *Deslfovibrio* and *Desulfobacter*.
  6. LA-inhibited propionate production by bacteria such as *Clostridium*, *Bacteriodes* and *Fusobacterium* by shifting their metabolic pathways. Although some of these species were detected in the LA-treated culture and their concentration was higher than in the control culture, no propionate was produced in LA-treated samples of the H<sub>2</sub> production experiment.
  7. The H<sub>2</sub> yield measured in this study is comparable to that reported from mutants of *Entrobacter*, which are blocked in biosynthetic pathways leading to production of organic acids and alcohols. In comparison to the 4 mol H<sub>2</sub>·mol<sup>-1</sup> glucose obtained from thermophilic *Thermotoga maritime* at 80°C in batch fermentation by Schroder *et al.* (1994), the yield in this study is reached 83% of the theoretical value under mesophilic conditions.
  8. Low pH alone is insufficient to suppress H<sub>2</sub> consumers or increase the yield of H<sub>2</sub>. The addition of 2,000 mg L<sup>-1</sup> LA significantly reduced the activity of H<sub>2</sub>-consuming microorganisms which, in turn, decreased H<sub>2</sub> consumption and increased its yield. The metabolic flux analysis model predicted that there would be no homoacetogenic

- H<sub>2</sub> consumption in LA-treated culture versus a maximum of 0.42 mol H<sub>2</sub>·mol<sup>-1</sup> glucose (18% of the maximum yield) in the glucose control cultures.
9. The results support the hypothesis that inhibition of methanogens by LCFAs is irreversible, and conflict with the reversible inhibition hypothesis. Furthermore, the present findings from the exposure of mixed cultures to LCFAs do not fully support the view that LCFAs inhibit the growth of Gram-positive bacteria, but do not inhibit Gram-negative bacteria. Some Gram-positive bacteria can survive inhibition by LCFAs; the dominance of *Clostridia*, *Bacillus* and other Gram-positive species in LCFAs-treated cultures contradicts the work from earlier reports.
  10. Species such as *Bacillus*, *Clostridium*, *Eubacterium*, *Propionibacteria*, and *Bacteroides* were detected in great abundance in the LA-control samples of both granular and flocculated cultures. Species such as *Bifidobacterium thermophilus*, *Actinobacillus salpingitidis*, *Flavobacterim*, *Anaeroplasma*, *Acinetobacter*, *Alicyclobacillus*, and *Corynebacterium* might be affiliated with LCFAs because they dominated LA-control samples of flocculated and granular cultures.
  11. The maximum H<sub>2</sub> yield observed in flocculated cultures (2.52±0.20 mol H<sub>2</sub>·mol<sup>-1</sup> glucose after a single glucose injection) was higher than that observed in granular culture (1.93±0.15 mol H<sub>2</sub>·mol<sup>-1</sup> glucose). Methanogenic activity was higher in granular culture compared to flocculated cultures. Thus, H<sub>2</sub> consumption by hydrogenotrophic methanogens was minimized in flocculated compared to granular cultures.
  12. The greater diversity of fermentative and acidogenic H<sub>2</sub>-producing bacteria (i.e. *Bacteroides* (52%), *Clostridium* (25%), and *Bacillus*, *Brevibacilus*, and *Clostridium*

(11%), and *Moorella thermoacetica* (5% detected in the flocculated cultures fed glucose and treated with LA accounted for the higher H<sub>2</sub> yield. The FBA analysis did not show any homoacetogenic H<sub>2</sub> consumption activity in the flocculated cultures fed glucose and incubated with LA despite the presence of homoacetogenic species such as *Moorella thermoacetica*. Conversely, FBA showed homoacetogenic H<sub>2</sub> consumption alongside greater methanogenic activity in the granular culture fed glucose and treated with LA. The dominant species detected were *Bacillus thermoleovorans* (37%), *Eubacterium* (19%), *Clostridium*, *Butyrivibrio*, *Fusobacterium*, and *Gloeobacter* sp. (6%), and *Bacteroides* and *Falvobacterium* (4%). Therefore, granular cultures are more favourable to H<sub>2</sub> consumption. The results suggest that LA affects the physiological and metabolic activity of microorganisms in the granular as well as the flocculated cultures. However, the inhibitory effects on H<sub>2</sub> consuming microorganisms are more predominant in the flocculated cultures.

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## **CHAPTER 10: ENGINEERING SIGNIFICANCE AND SUGGESTIONS FOR FUTURE RESEARCH**

This research provides data that elucidate the dynamics of the microbial community in hydrogen ( $H_2$ ) producing mixed culture under the stress of LCFAs and low pH. The work addresses the issue of controlling  $H_2$  metabolism in mixed anaerobic culture using sustainable and renewable natural organics to improve a sustainable process that treats agricultural waste and produces  $H_2$  as a clean fuel. The findings characterize aspects of the  $H_2$  producing mixed culture where control mechanisms could be applied to increase the  $H_2$  yield by directing electron flux towards  $H_2$  rather than other by-products, and to minimize  $H_2$  consumption.

The results from phase I of this study demonstrate that LA can overcome variations in the microbial communities from different sources and bring the  $H_2$  yields to comparable levels. Therefore, LCFAs such as LA can provide natural alternatives to toxic chemical inhibitors used to inhibit  $H_2$  consumers, shift the metabolic pathway, and direct the electron flux towards  $H_2$ , acetate, and butyrate while decreasing the production of more reduced by-products such as lactate, propionate, and alcohols. In comparison to other evidence from other pretreatment methods, LCFAs satisfy all the prerequisites for large-scale industrial application such as low-cost, environmentally friendly, sustainable, renewable, and efficient at maximizing the  $H_2$  yield. The flux balanced analysis proved to be an important tool which can be used to enable insight into the intercellular metabolite fluxes and to explain the  $H_2$ -producing and consuming routes.

In phase II, the results show that long-term adaptation of the mixed anaerobic cultures to glucose changed the composition of microbial populations and affected their activities. The twelve months of adaptation selected acidogens over other trophic groups and increased H<sub>2</sub> yield. Species such as *Clostridia*, *Bacillus*, *Geobacter*, *Entrobacter*, *Erwinia*, *E. Coli*, *Eubacterium*, and *Klebsiella* dominated after adaptation whereas *Lactobacillus*, *Baceroides*, and sulfate-reducing bacteria declined. This study provides kinetic and microbiological data on the effect of long-term adaptation, and opens the opportunity for further research to answer the question of whether the observed improvements are due to genetic changes, enzyme activities or both? Another issue that requires further experimental work is investigation of the effect of long-term adaptation of the culture to LCFAs on H<sub>2</sub> production in both batch and continuous reactors. Genetic and enzyme assays could be conducted to assess the possibility of adaptation to LCFAs by methanogens.

The multidisciplinary approach of combining kinetic, molecular biology, metabolic flux analysis, multivariate statistical analysis, and H<sub>2</sub> production methods to this particular research application is unique to this study. Integrating these data sources might shift the research framework from descriptive to predictive. This approach could be developed and optimized as a monitoring and control protocol for large-scale applications to predict process imbalances.

The results from phase III suggest that low pH alone is not sufficient to minimize the consumption of H<sub>2</sub> by methanogens and homoacetogens; concurrently, it clearly demonstrates that LA inhibits methanogens completely and reduces the activity of homoacetogens significantly during H<sub>2</sub> dark fermentation. The findings are important

because they provide quantification of H<sub>2</sub> consumption in H<sub>2</sub> production studies under different conditions. This information has not been reported in the literature. Moreover, the findings provide a method to greatly minimize H<sub>2</sub> consumption. The experimental work also revealed the difficulty of assessing the kinetics of the degradation of volatile fatty acids under stress conditions (2,000 mg L<sup>-1</sup> LA and low pH) because of the very slow degradation rate. Another methodological issue that became apparent during acetate degradation under these stress conditions was the difficulty of differentiating between acetate injected and acetate produced as a by-product of LA degradation and further research using labeled carbon compounds is suggested as a possible solution.

The results from phase IV is directed to using PA as another potential stressing agent which can support high H<sub>2</sub> yield (in comparison to MA and LUA). However, note PA is not as strong as LA. Although LUA (C12:0) is known as a strong inhibitor to methanogens it did not improve the H<sub>2</sub> yield in this study and further optimization experiments are needed for PA and LUA.

In phase V the findings demonstrate that the H<sub>2</sub> yields from flocculated cultures are better than the yields from granular cultures in a long-term incubation study (0 to 30 days). These findings are important because, in contrast to all other reported pretreatments, incubation with LCFAs induced higher H<sub>2</sub> yields in flocculated cultures than in granular culture. The outcomes for this phase of the study showed that 2,000 mg L<sup>-1</sup> of LA can sustain high H<sub>2</sub> yield for a minimum of 30 days. This is an important finding because it means that smaller quantities of LA will be required and that no toxic effluent will be discharged in comparison with other toxic inhibitors such as BESA and chloroform. Furthermore, combining the findings from phases IV and V that PA was

more effective than other LCFAs shorter than C18 (phase IV) and that PA was the main by-product of LA degradation (phase V) suggests that further research is warranted to investigate and optimize the use of PA to improve H<sub>2</sub> yield in mixed cultures.

All studies carried out for this research project were in laboratory-scale batch reactors. Pilot studies in batch and continuous reactors are suggested as a necessary first step to evaluate the potential of LCFAs in improving H<sub>2</sub> yield in large-scale applications. Moreover, the approach used in the current research could be further developed, assessed and applied to process control for the large-scale application.

## APPENDIX (A): CALIBRATION CURVES

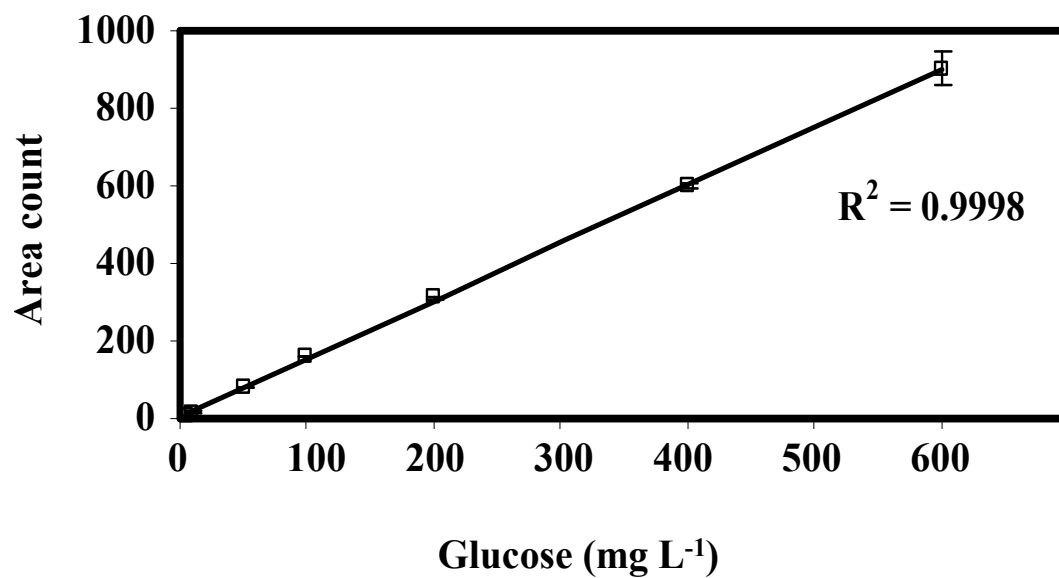


Figure A.1 Glucose calibration curve.

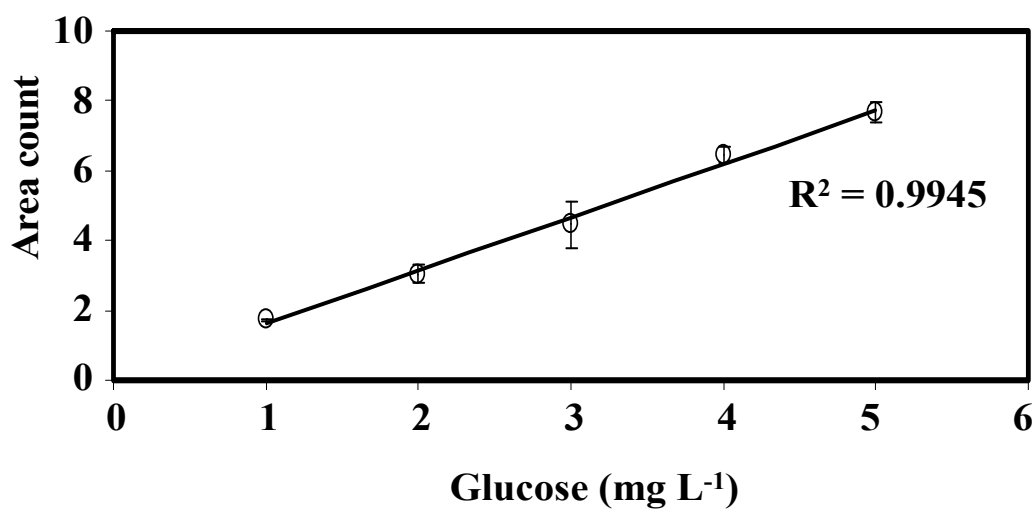


Figure A.2 Glucose detection limit curve.



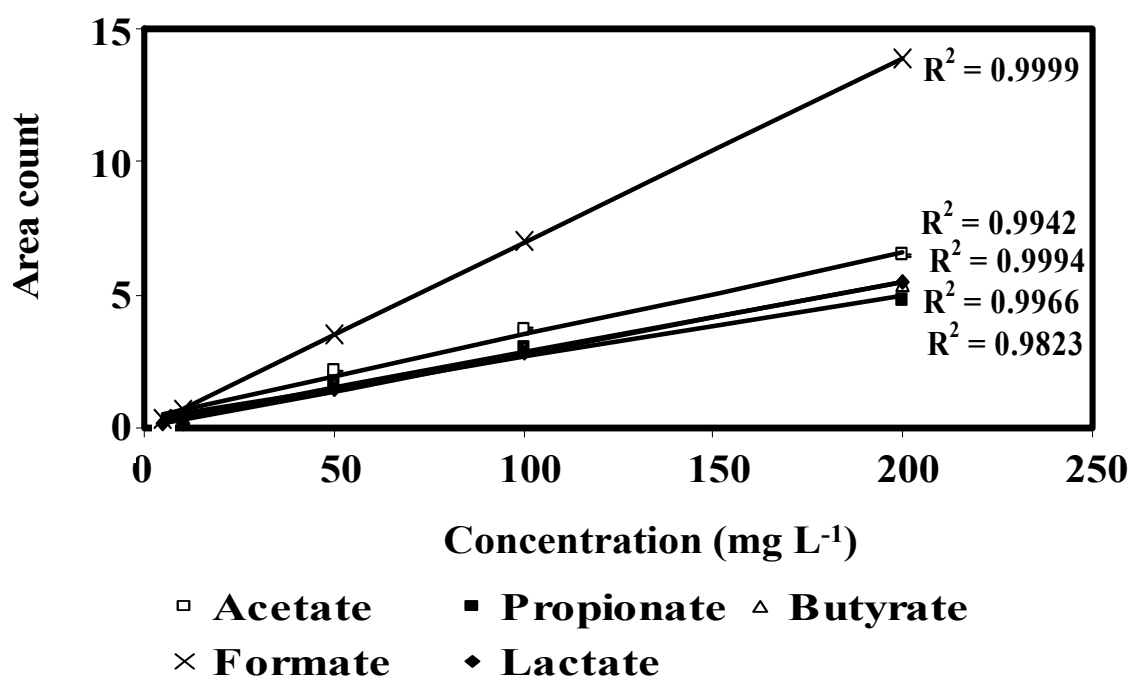


Figure A.3 Volatile fatty acids calibration curve.

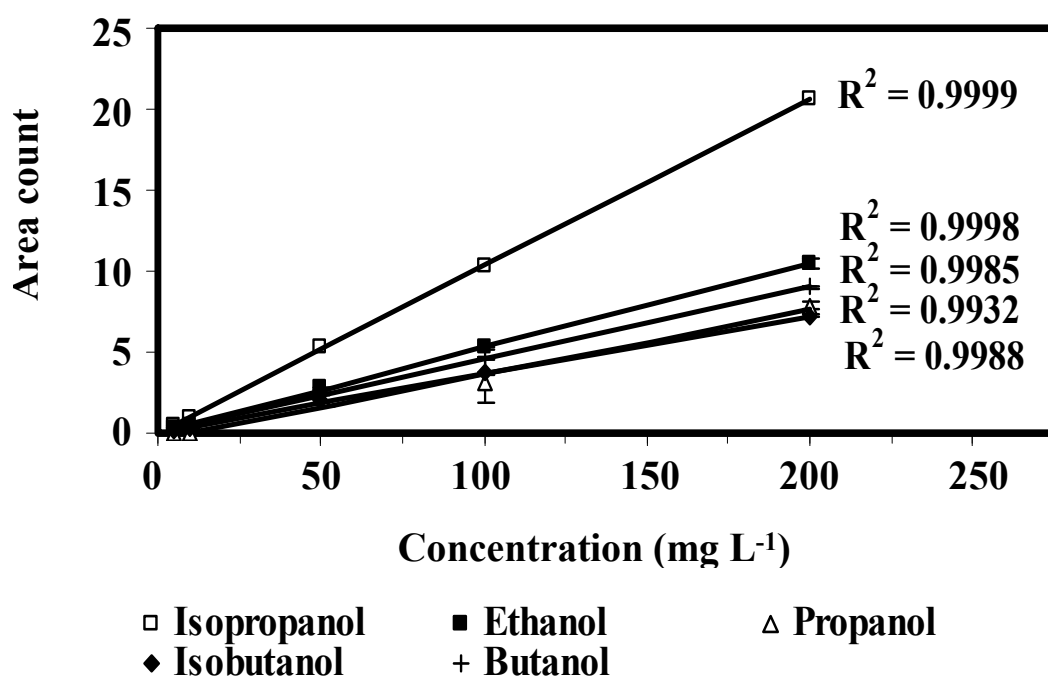


Figure A.4 Alcohols calibration curve.

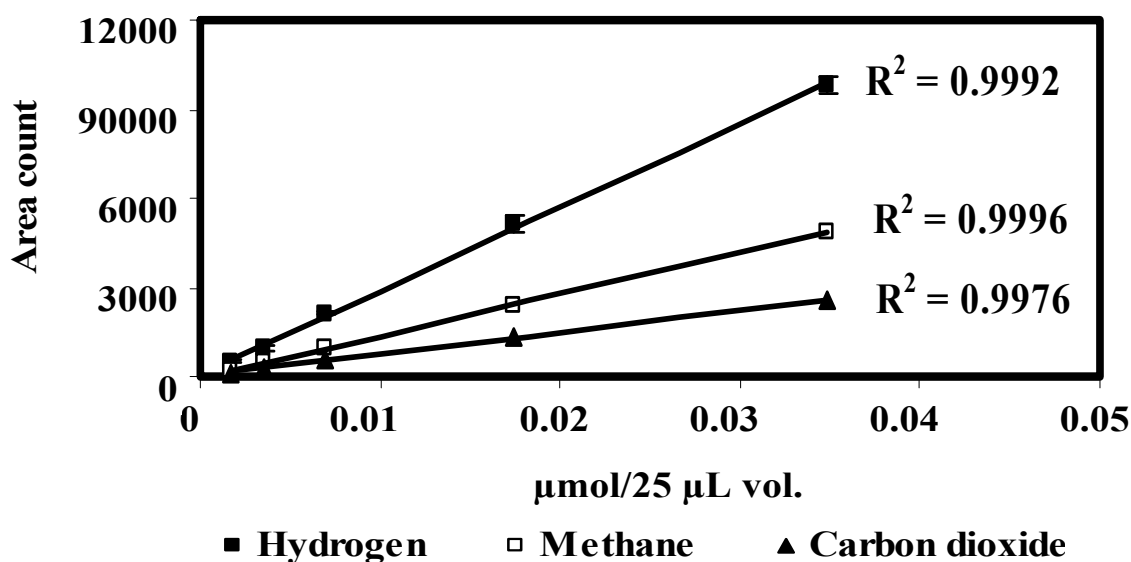


Figure A.5 Gases calibration curves.

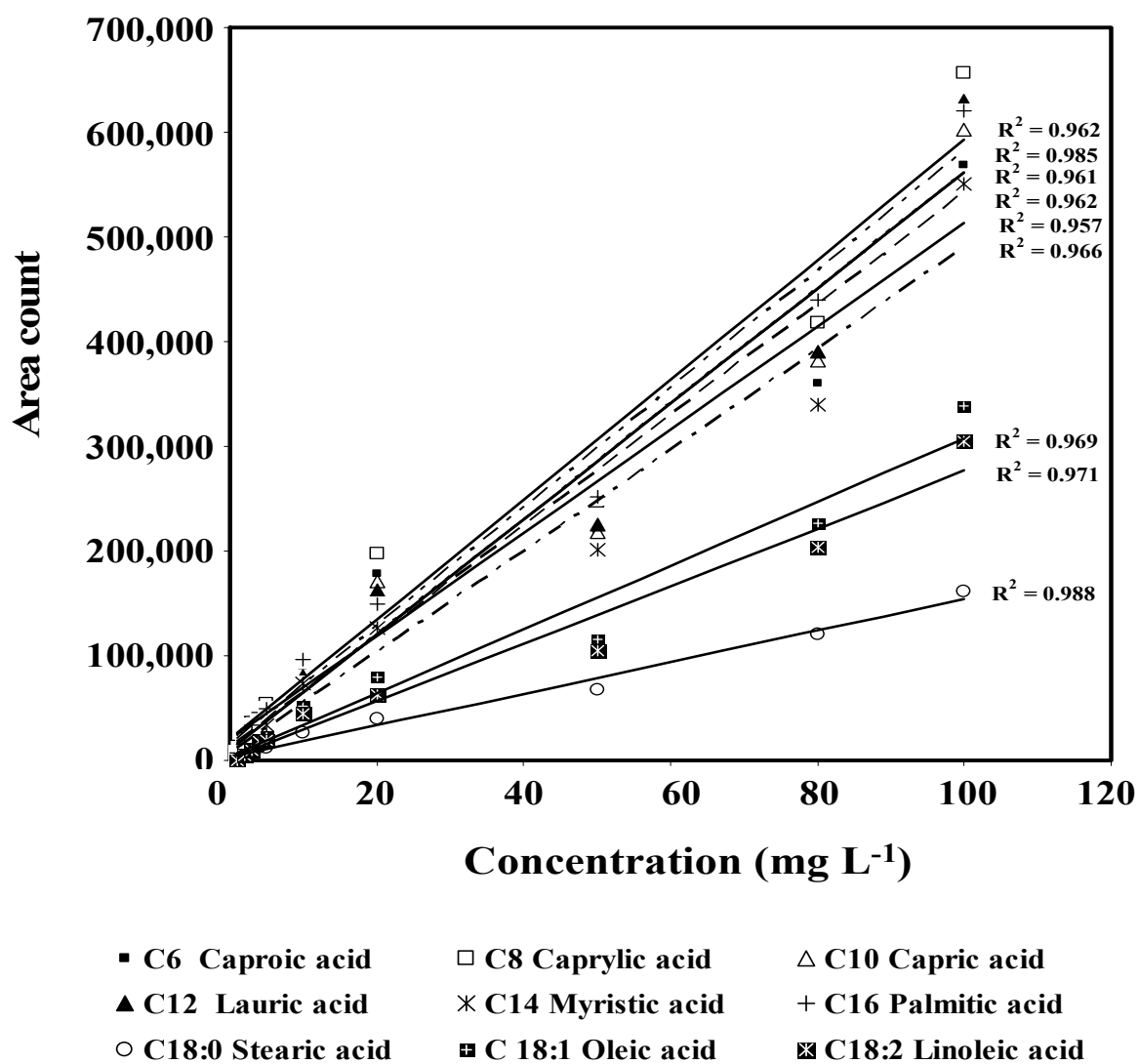


Figure A.6 Long chain fatty acids calibration curves.

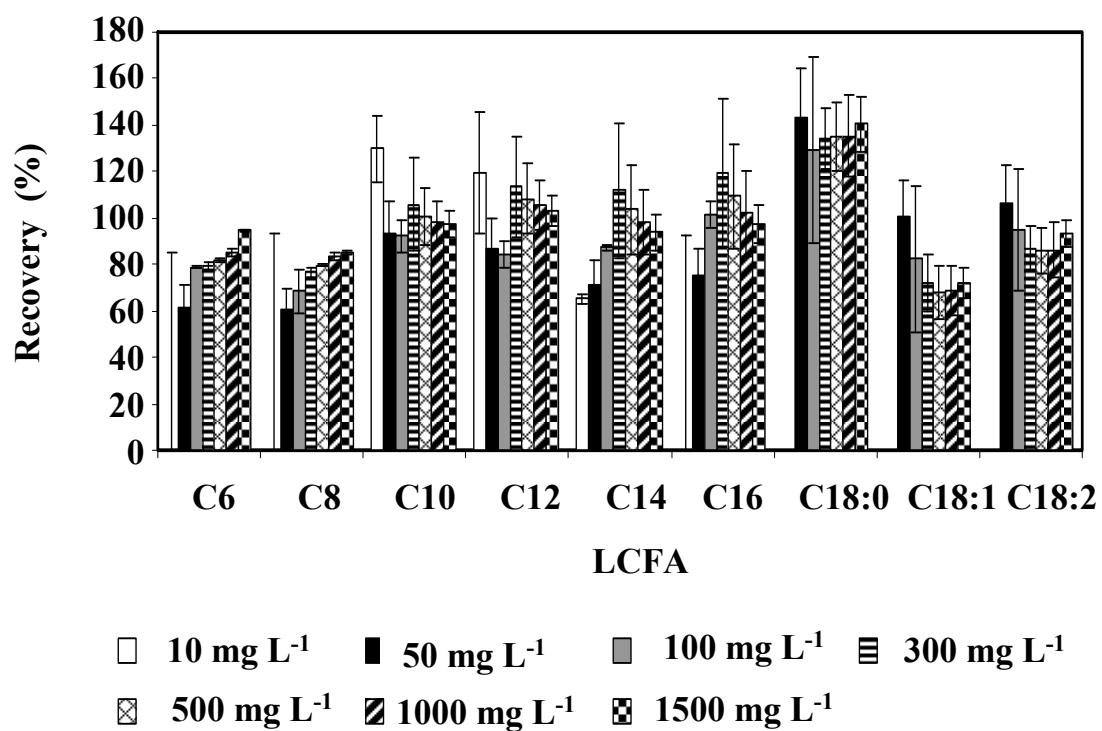


Figure A.7 Long chain fatty acids extraction recovery plot.

## Appendix (B): Reactor characterization (37 °C)

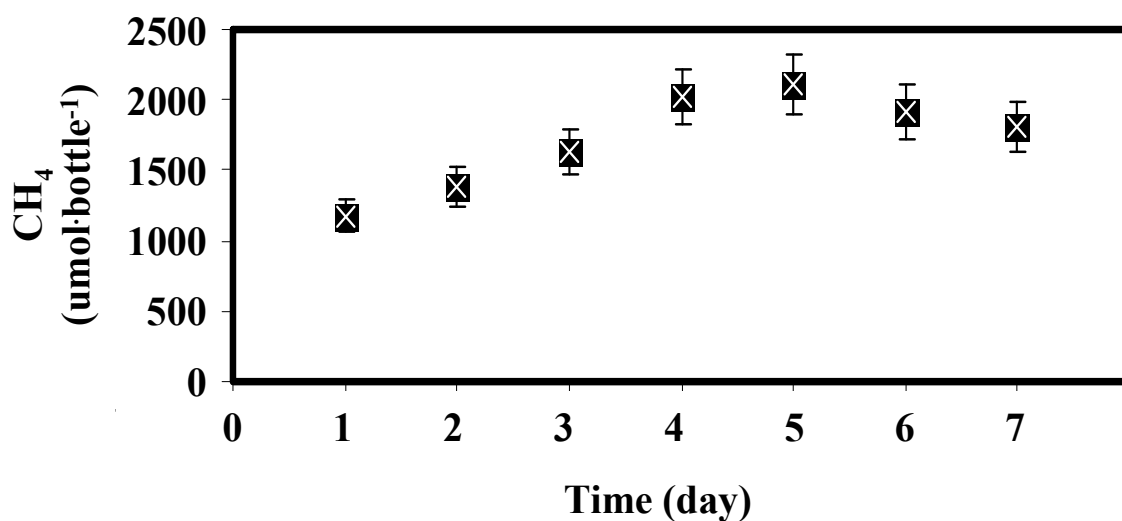


Figure B.1 Methane production profile in cultures using inocula from the reactor at pH 7.6. (Values shown are mean±standard deviation for triplicate samples).

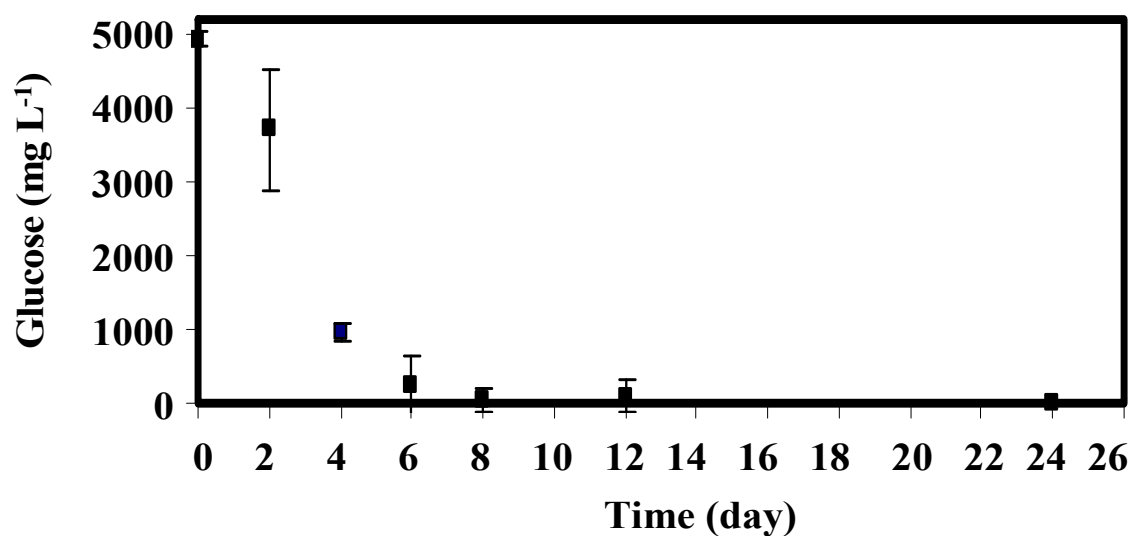


Figure B.2 Glucose degradation profile in cultures using inocula from the reactor at pH 7.6 (Values shown are mean±standard deviation for triplicate samples).

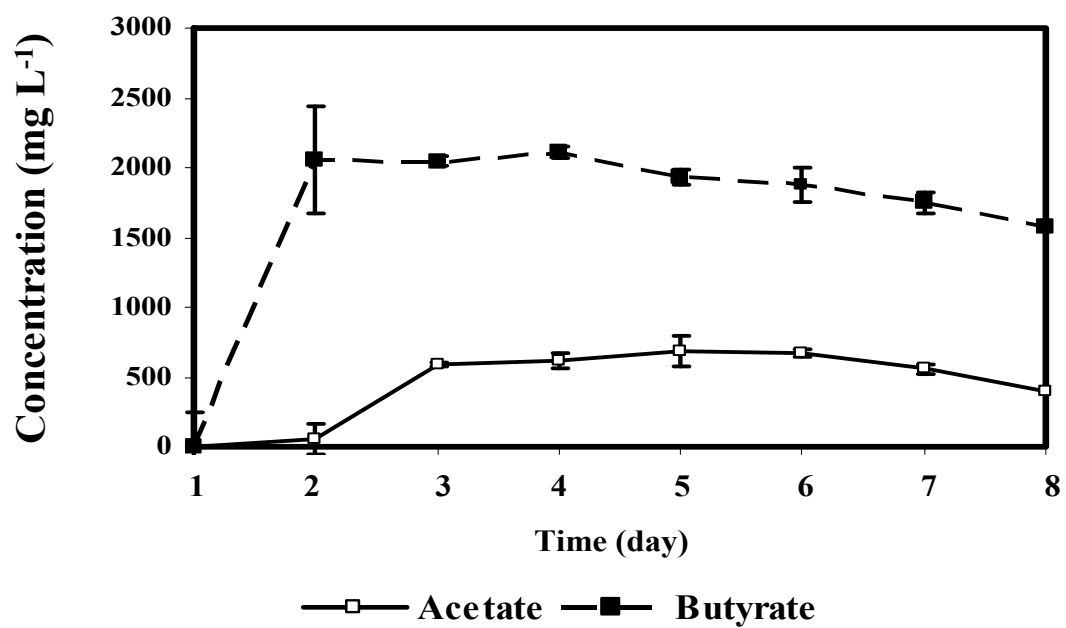


Figure B.3 Volatile fatty acids production profiles in inocula from the reactor at pH 7.6 (Values shown are mean $\pm$ standard deviation for triplicate samples).

## APPENDIX (C): EQUATIONS USED IN CALCULATING $k_a$ AND $X_a$

The Monod equations for consumption of a specific substrate and microbial growth in a batch system, neglecting decay, are as follows:

$$\frac{dS}{dt} = \left( \frac{k_a S}{K_s + S} \right) X_a \quad (C.1)$$

$$\frac{dX_a}{dt} = -Y \frac{dS}{dt} \quad (C.2)$$

Where:

S = substrate concentration, mg COD L<sup>-1</sup>

T= time, day

$k_a$  = maximum specific substrate utilization rate, mg substrate as COD mg active biomass as COD/day.

$K_s$  = half-saturation constant, mg COD L<sup>-1</sup>

$X_a$  = active biomass concentration which consumes the substrate, mg COD L<sup>-1</sup>.

$Y$  = microbial growth yield, mg active biomass as COD per mg substrate consumed as COD

When  $S \gg K_s$ , Eq. C.1 becomes zero order with respect to S, and can be simplified to:

$$\frac{dS}{dt} = -k_a X_a \quad (C.3)$$

Or

$$X_a = -\frac{dS / dt}{k_a} \quad (C.4)$$

Substituting equation C.3 into C.2 gives:

$$\frac{dX_a}{dt} = k_a Y X_a \quad (\text{C.5})$$

By integrating Eq. C.5;

$$\ln X_a - \ln X_{a0} = k_a Y t \quad (\text{C.6})$$

Similarly, substituting Eq. C.4 into Eq. C.6 gives;

$$\ln \left| \frac{dS}{dt} \right| = k_a Y t + \ln \left| \frac{dS}{dt} \right|_{t=0} \quad (\text{C.7})$$

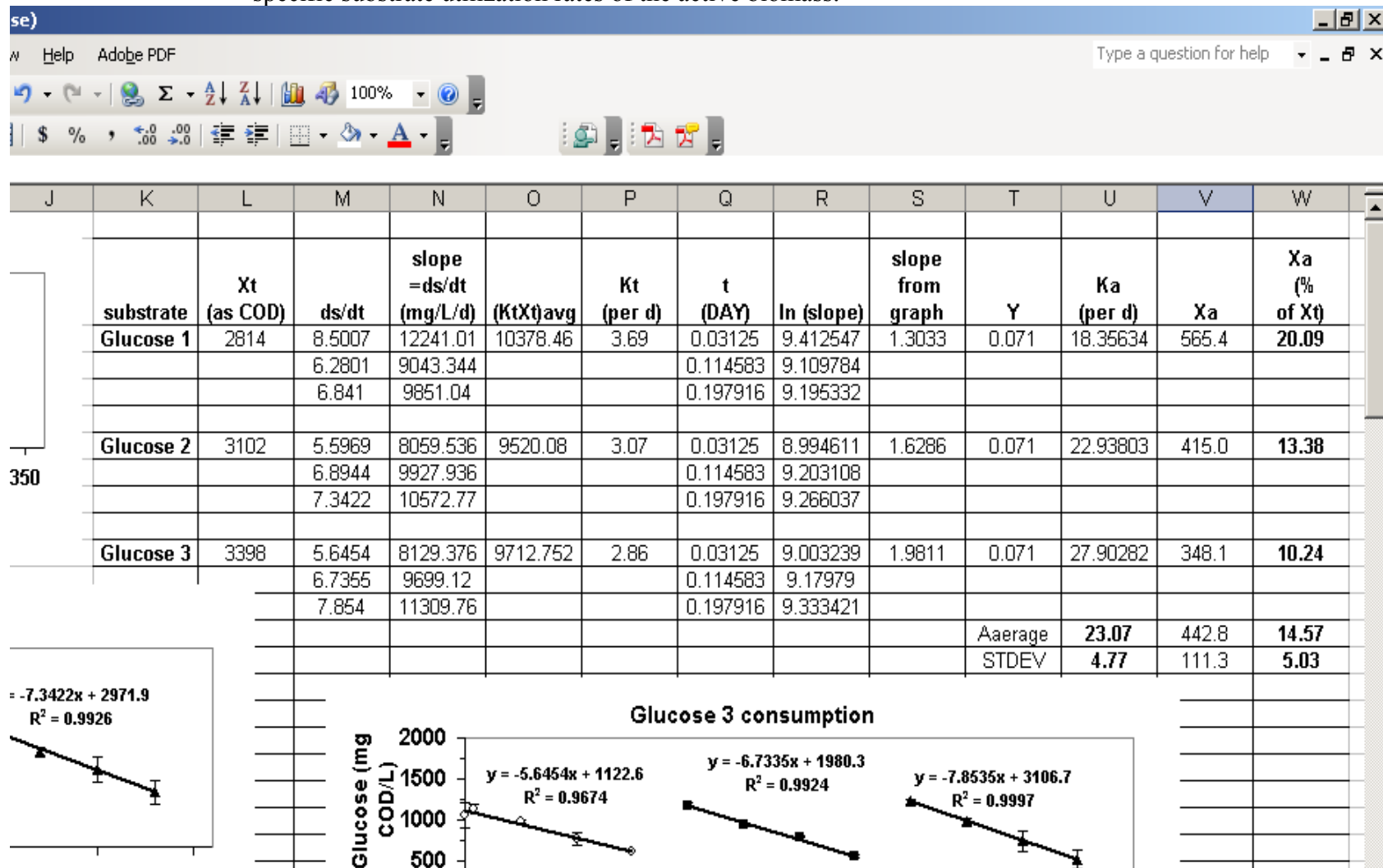
The slope of the plot of  $\ln \left| \frac{dS}{dt} \right|$  vs. time is  $k_a$ . Hence the value of  $k_a$  can be calculated by assuming an appropriate value for the yield,  $Y$ .  $X_a$  can then be calculated as:

$$X_a = -\frac{k_t X_t}{k_a} \quad (\text{C.8})$$

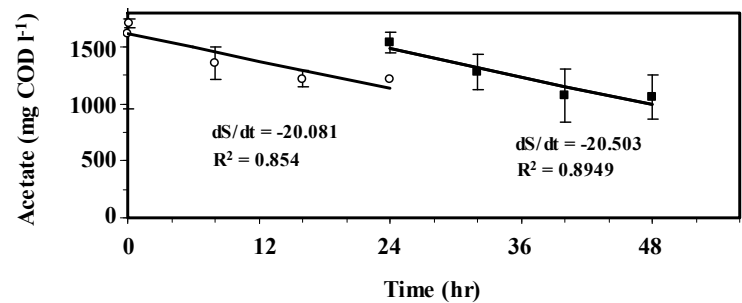
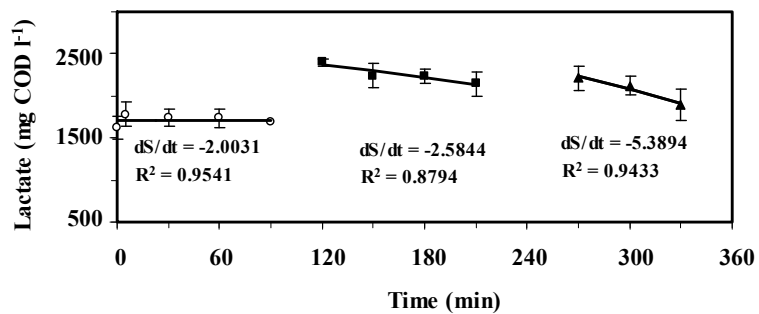
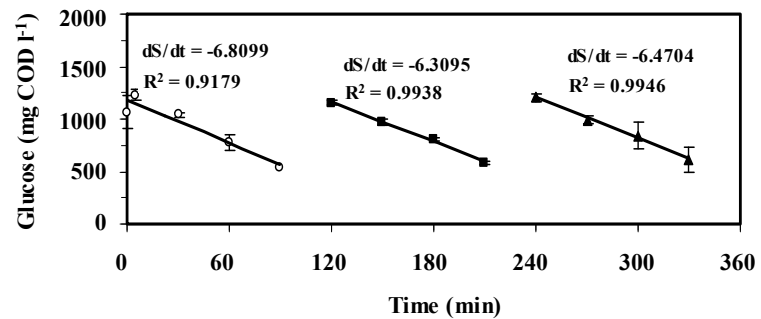
Where  $k_t$  and  $X_t$  are based on the total biomass measured as VSS. if the total biomass measured is approximately constant (resulting in a linear substrate depletion curve),  $k_t$  can be measured as the slope of the substrate depletion curve divided by  $X_t$  which is the measured biomass as VSS.

$$k_t = -\frac{dS/dt}{X_t} \quad (\text{C.9})$$

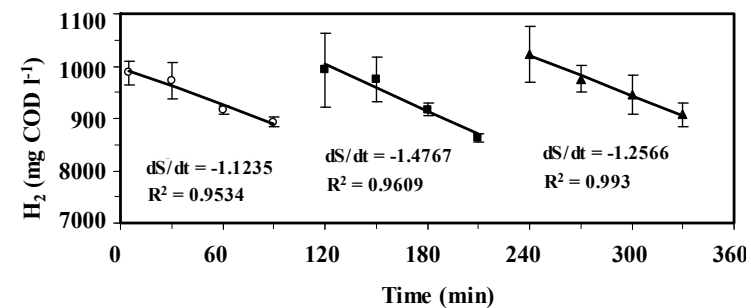
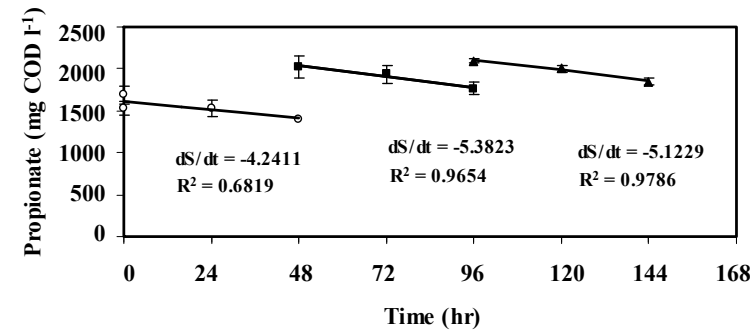
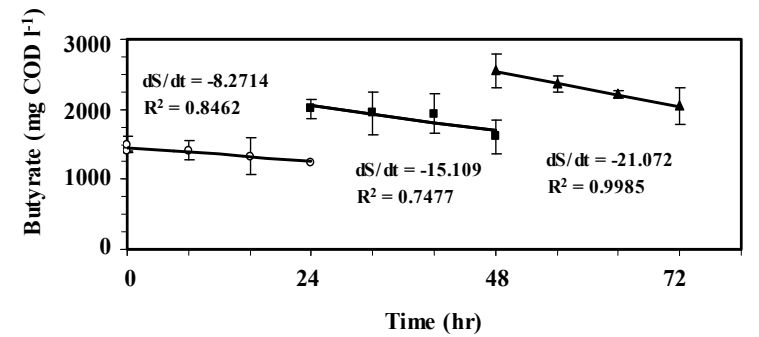
Figure C.1 Screen shot of the spreadsheet developed for calculating percentage and maximum specific substrate utilization rates of the active biomass.



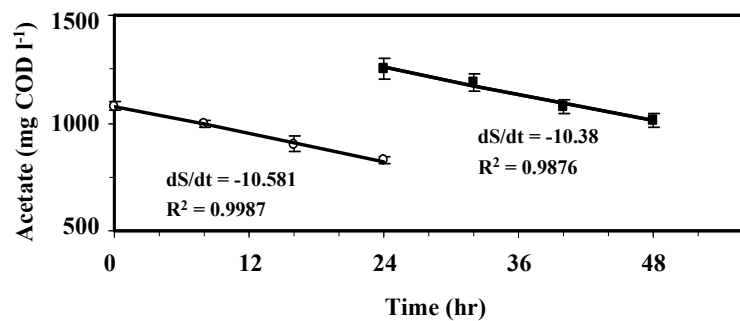
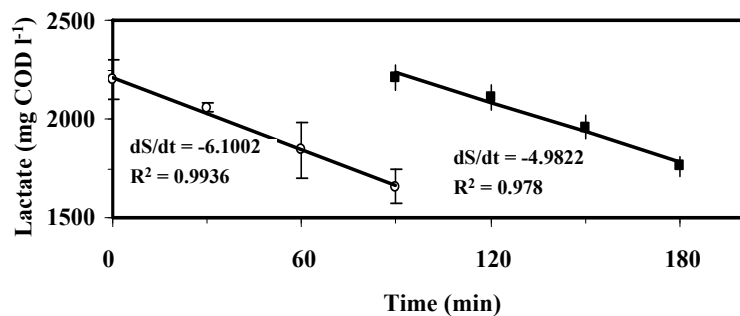
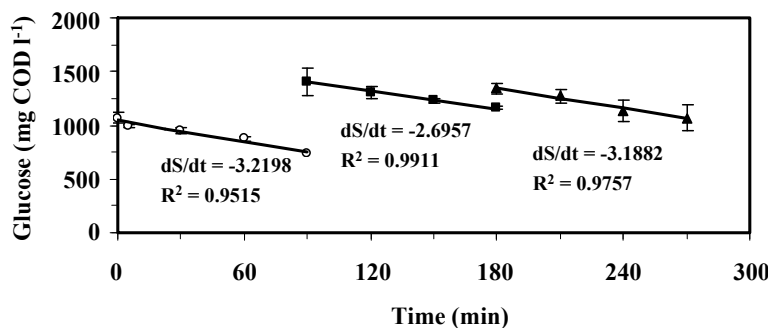




○ 1st injection    ■ 2nd injection    ▲ 3rd injection  
 Figure C-2 Kinetic of Culture A after adaptation to glucose.

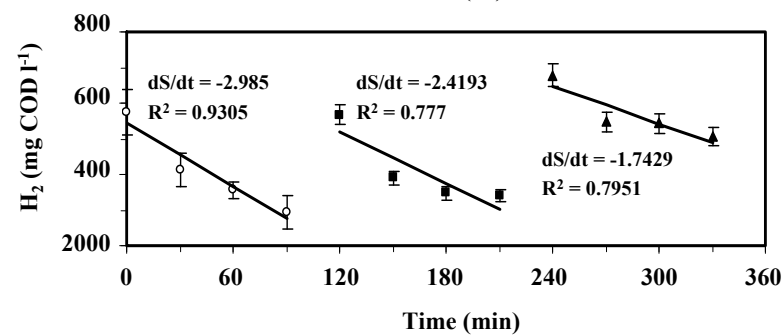
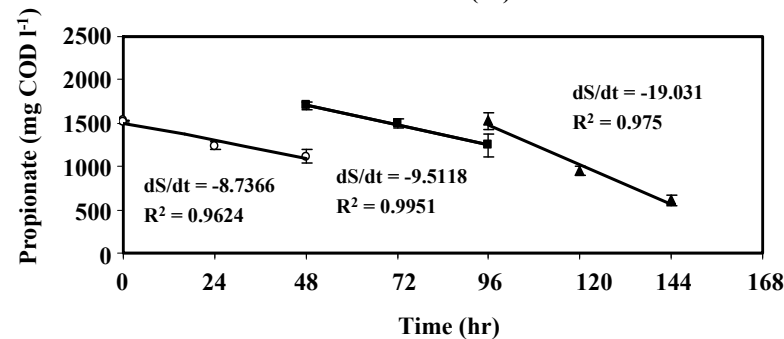
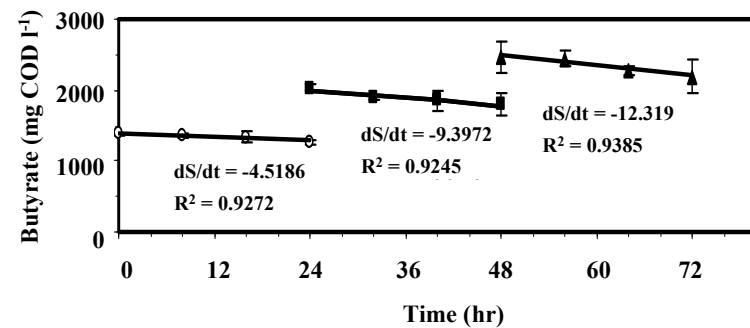


○ 1st injection    ■ 2nd injection    ▲ 3rd injection  
 Figure C-3 Kinetic of Culture A after adaptation to glucose.



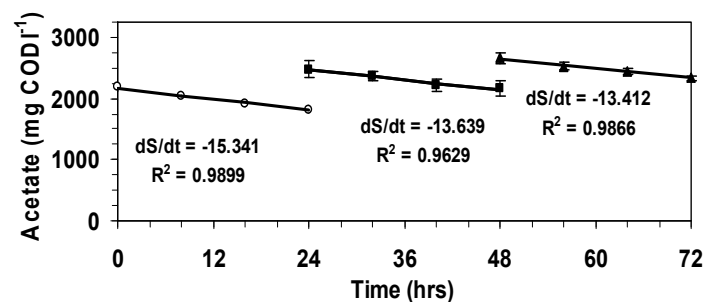
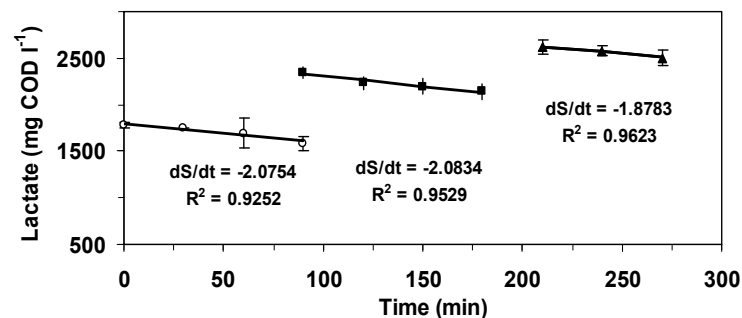
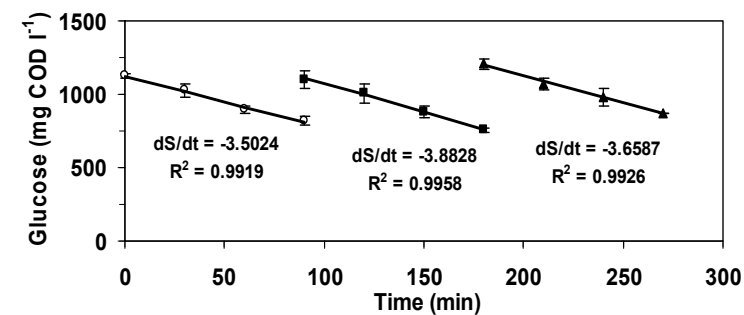
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Figure C-4 Kinetic of culture A before adaptation to glucose.



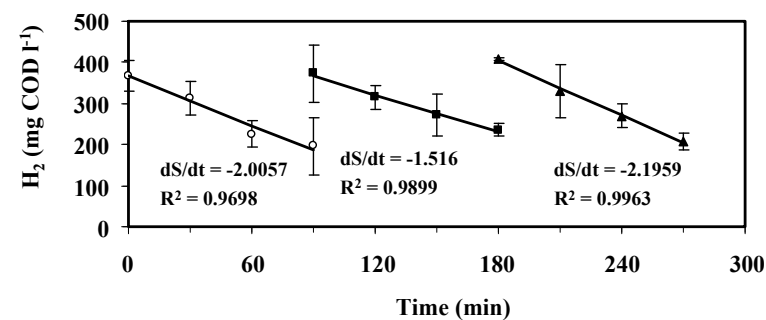
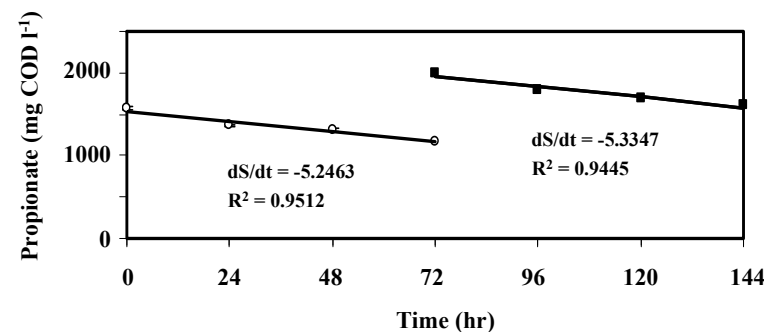
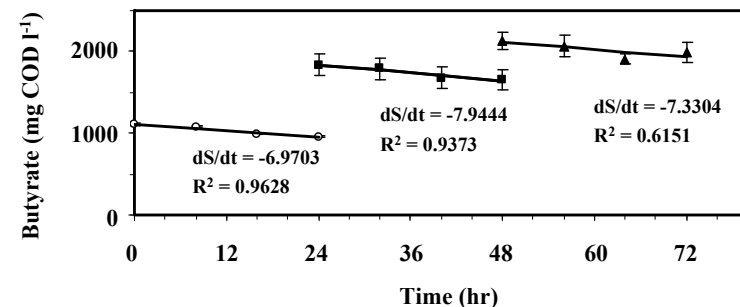
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Figure C-5 Kinetic of culture A before adaptation to glucose.



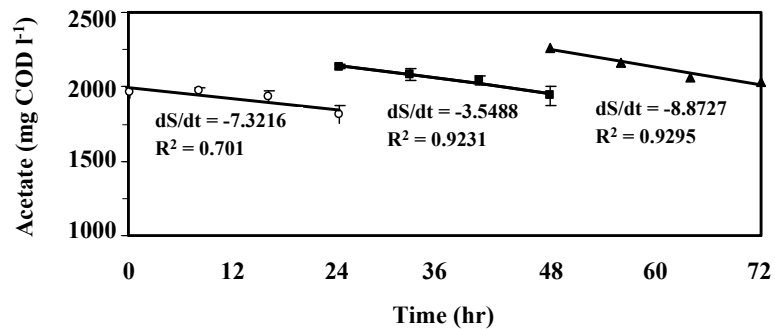
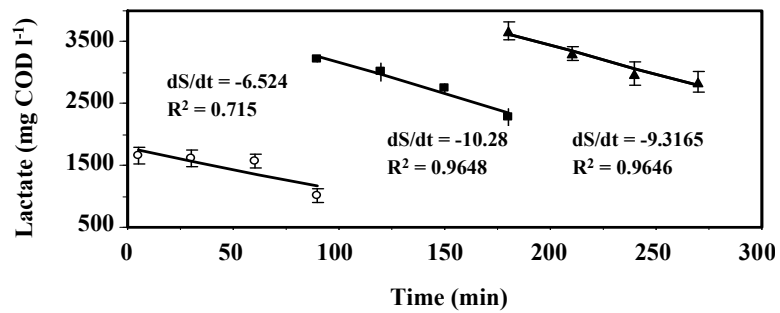
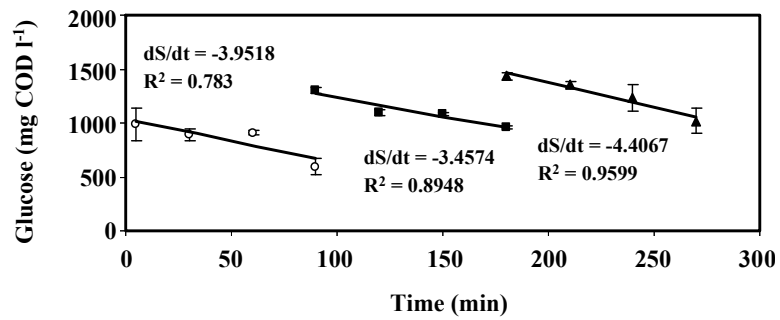
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Figure C-6 Kinetic of culture B after adaptation to glucose.

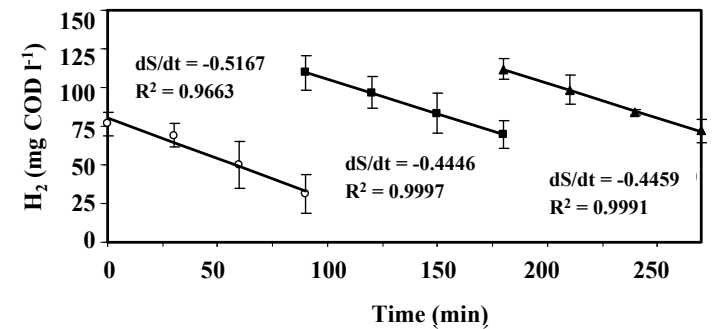
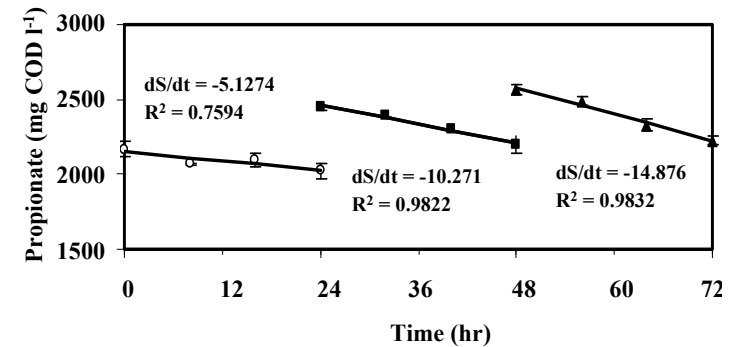
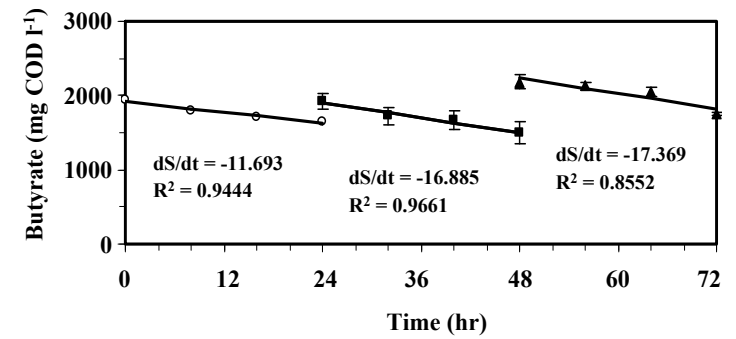


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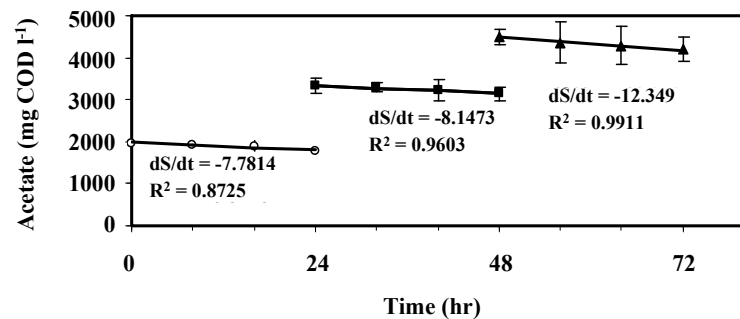
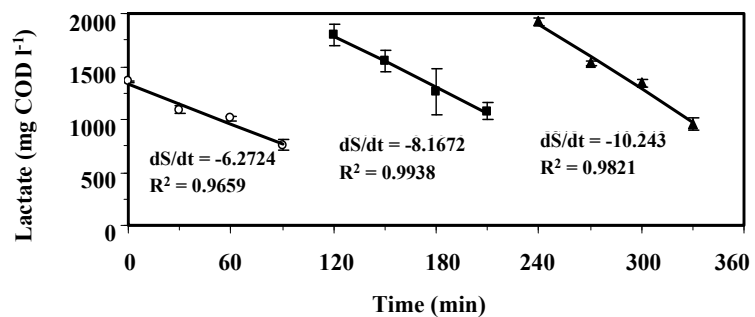
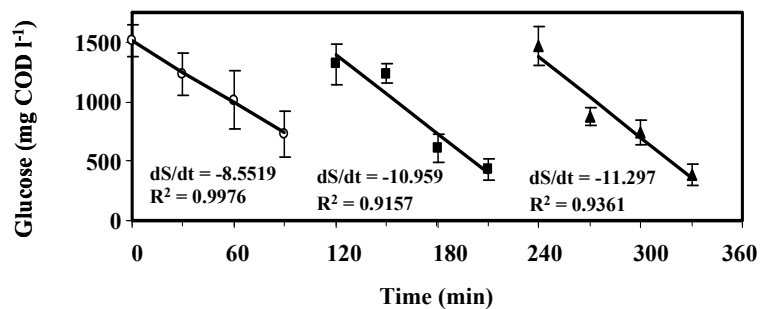
Figure C-7 Kinetic of culture B after adaptation to glucose.



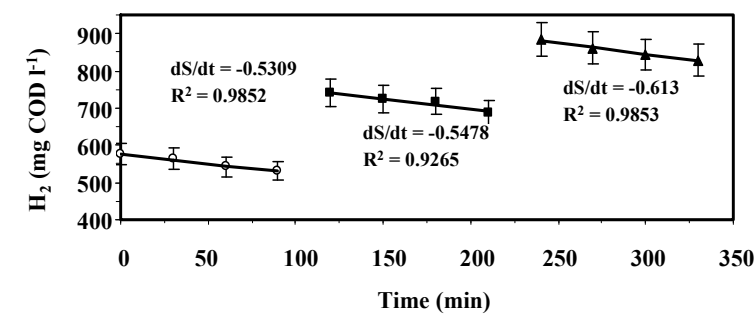
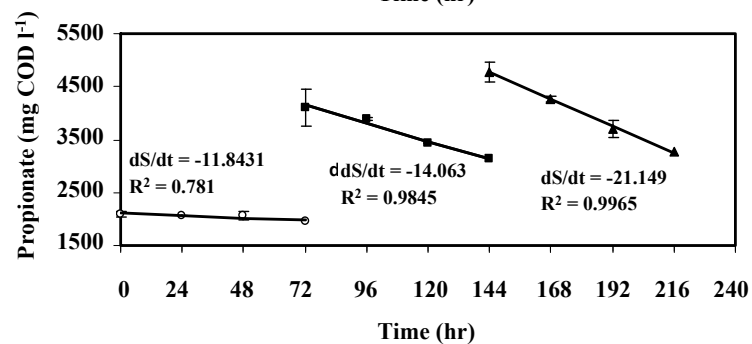
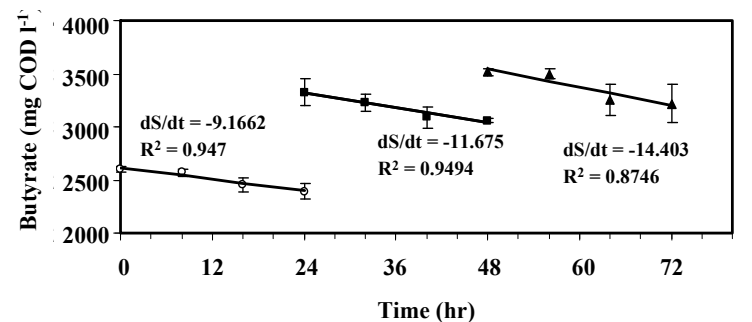
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Figure C-8 Kinetic of culture B before adaptation to glucose.



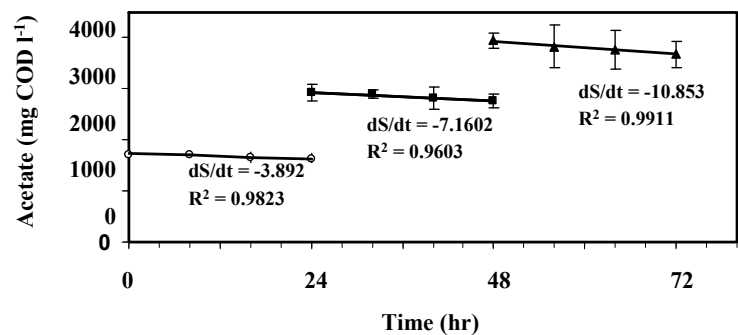
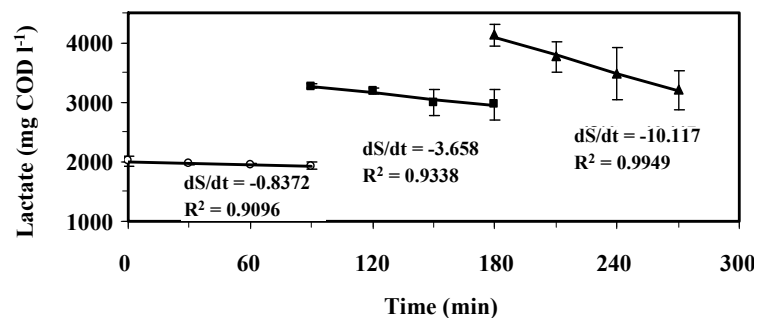
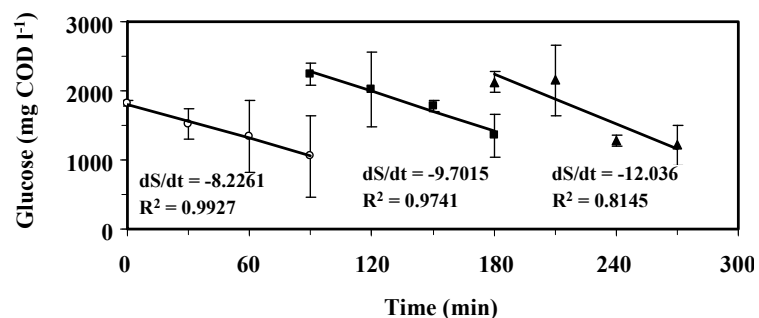
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Figure C-9 Kinetic of culture B before adaptation to glucose.



○ 1st injection    ■ 2nd injection    ▲ 3rd injection  
Figure C-10 Kinetic of culture C after adaptation to glucose.

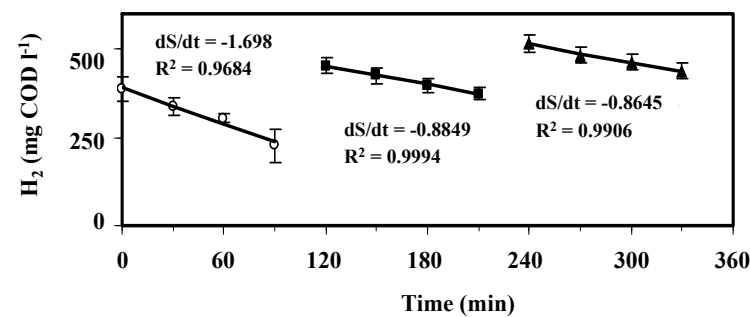
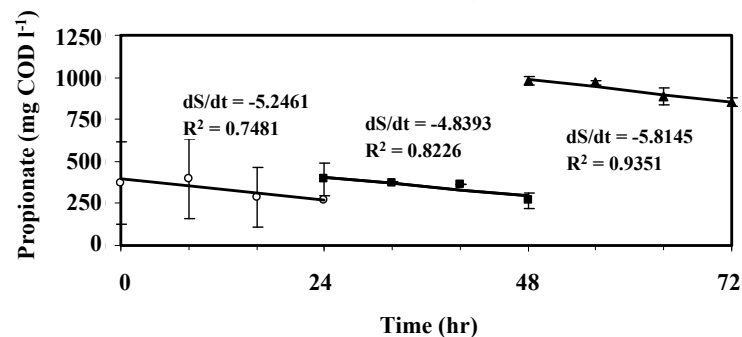
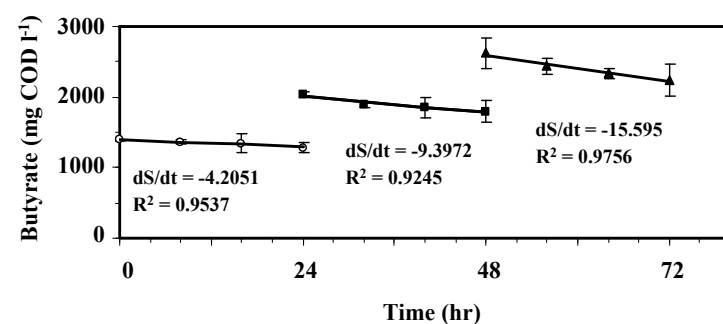


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Figure C-11 Kinetic of culture C after adaptation to glucose.



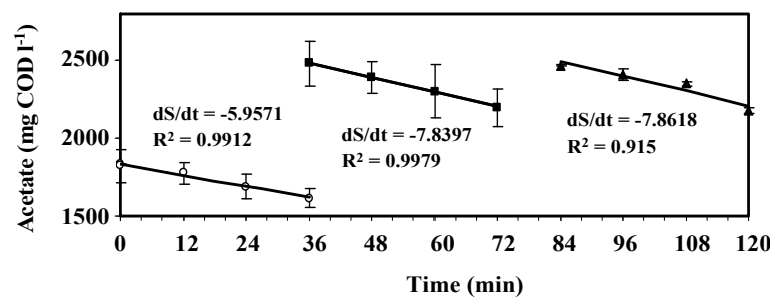
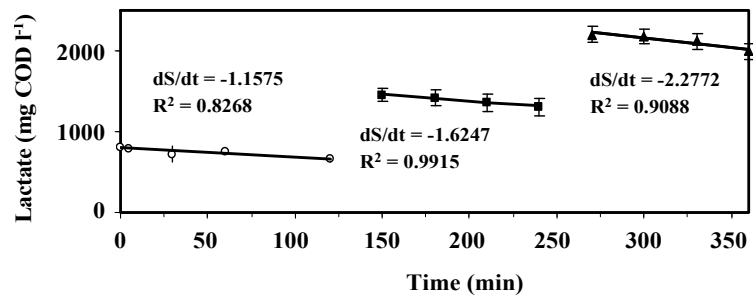
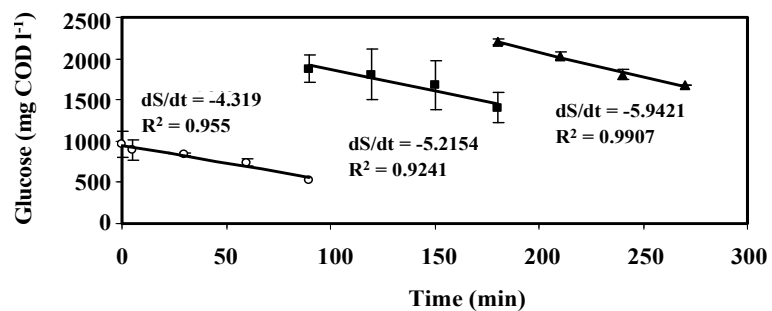
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Figure C-12 Kinetic of culture C before adaptation to glucose.



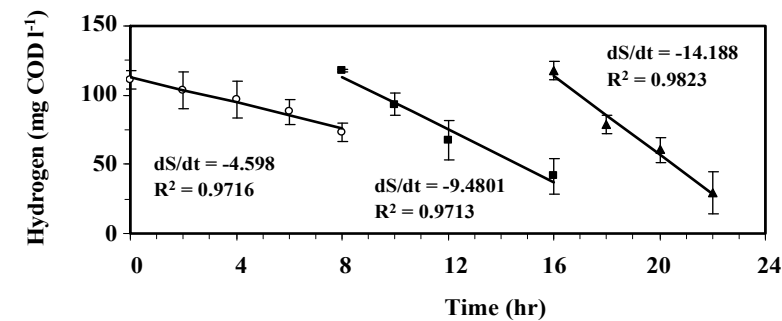
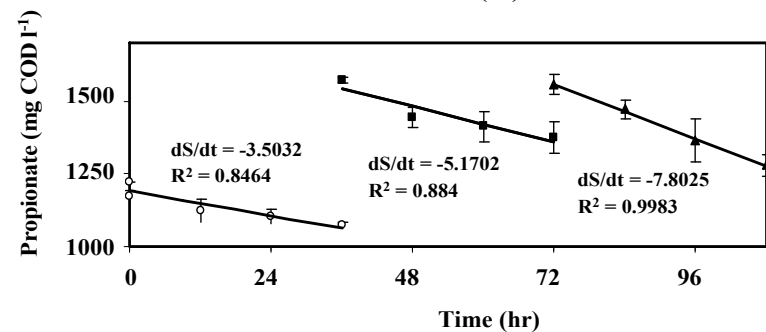
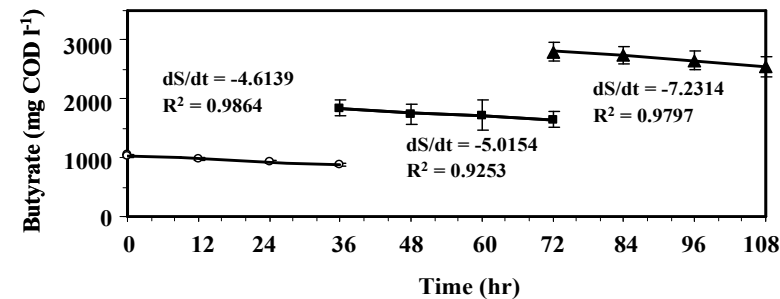
○ 1st injection    ■ 2nd injection    ▲ 3rd injection

Figure C-13 Kinetic of culture C before adaptation to glucose.



○ 1st injection    ■ 2nd injection    ▲ 3rd injection

Figure C-14 Kinetic of culture B after adaptation to glucose with an initial pH 5.0.

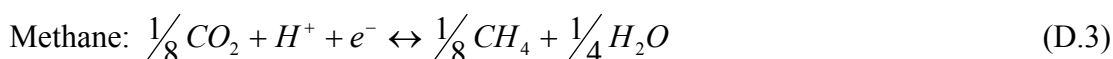


○ 1st injection    ■ 2nd injection    ▲ 3rd injection

Figure C-15 Kinetic of culture B after adaptation to glucose with an initial pH 5.0.

## APPENDIX (D): ELECTRON MASS BALANCE SAMPLE CALCULATIONS

The electron ( $e^-$ ) mass balance takes into account the electron equivalents available in the glucose and the by-products of fermentation. It does not include the LCFA parent compound or its by-products in 8 to 10 days incubation experiments in Chapters 4, 5, 6, and 7. Nevertheless, in Chapter 8 where longer incubation periods (up to 30 days) have been investigated, the electrons contributed from the degradation of LCFAs were subtracted from the total electrons contributed from glucose and LCFAs. This was achieved by subtracting the electrons present in acetate derived from the  $\beta$ -oxidation of LCFAs while considering the percentage of electron in acetate as a result of glucose degradation. The electron balance assumes that 10 % of the electrons from glucose degradation are synthesized into biomass. The number of electron equivalents ( $e^-$  equiv) available in glucose and by-products was determined from the half reactions:



Then, mole of acetate gives 8 mole of electron equivalents, mole of hydrogen gives 2 mole of  $e^-$  equiv mole of methane gives 8 mole of  $e^-$  equiv, mole of glucose gives 24 mole of  $e^-$  equiv, and so on.

The molar concentrations of substrates and by-products were converted to electron equivalents based on the stated relationships and were related as a percentage based on the initial amounts of substrate injected.

$$\text{Electron mass balance (\%)} = \frac{e^- \text{ in by-products} + e^- \text{ in biomass} + e^- \text{ in glucose remaining}}{e^- \text{ in glucose initially injected}} * 100 \quad (\text{D.4})$$

Compound	Concentration (mg L <sup>-1</sup> )	Concentration (mM)	$e^-$ equiv available per mol	$e^-$ equiv In	$e^-$ equiv Out
Glucose	5,000	1.388	24	33.333	
H <sub>2</sub>		0.961	2		1.922
CH <sub>4</sub>		0.566	8		4.528
Lactate	0		12		
Acetate	924.804	0.783	8		6.269
Propionate	1186.117	0.812	14		11.220
Butyrate			20		
Formate	0		2		
Isopropanol	36.103		18		0.541
Ethanol	0		12		
Propanol	315.068		18		4.718
Isobutanol	67.854		22		0.828
Butanol	0		22		
Biomass			---		3.333
<b>Total</b>				33.333	33.356
<b>%</b>	33.35/33.33 *100 = 100 %				

$e^-$  equiv = electron equivalent



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